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Effects of membrane lipid composition on the kinetics of cholesterol exchange between lipoproteins and different species of red blood cells

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To better understand the effects of plasma membrane structure on the kinetics of cellular cholesterol efflux to extracellular lipoprotein particles, the influence of plasma membrane sphingomyelin (SM) on the kinetics of cholesterol exchange was examined in both a model membrane system comprised of egg SM/egg phosphatidylcholine (PC) unilamellar vesicles and in various types of mammalian red blood cells (RBC) containing differing levels of SM. The kinetics and mechanism of the bidirectional flux of unesterified cholesterol (FC) between RBC and lipoproteins were established by using human RBC (labeled with [^{14}C]FC) incubated with varying concentrations of human [^{3}H]FC high-density lipoprotein (HDL_3) or [^{3}H]FC low-density lipoprotein (LDL). A maximal rate constant for FC efflux was obtained when the lipoprotein FC was in excess (6-fold and 15-fold, for HDL_3 and LDL, respectively) of RBC FC; under this condition, the rate-limiting step is desorption of cholesterol molecules from the RBC membrane into the extracellular aqueous phase. At 37 °C, the halftime ($t_{1/2}$) for efflux was 4.6 ± 0.6 h for HDL_3 and 6.2 ± 0.2 h for LDL; FC efflux exhibited first-order kinetics and the RBC FC comprised a single kinetic pool. To investigate the effect of different membrane SM/PC ratios on the rate of FC desorption from the plasma membrane, the kinetics of cholesterol efflux from bovine RBC (5:1, w/w ratio of SM/PC), human RBC (1:1 ratio), rabbit RBC (0.6:1 ratio) and rat RBC (0.3:1 ratio) were compared. With excess HDL_3 present, bovine, rabbit, and rat RBC exhibited $t_{1/2}$ of 5.5 ± 0.8, 4.0 ± 0.2, and 3.7 ± 0.6 h, respectively, for cholesterol efflux. Changing the ratio from 0.3:1 to 3:1 in egg SM/egg PC small unilamellar vesicles increased the $t_{1/2}$ for cholesterol efflux at 45 °C from 1.1 to 6.9 h. The results described in this paper suggest that increasing membrane SM content raises the $t_{1/2}$ for cholesterol exchange in both the RBC plasma membrane and in simple mixed SM/PC bilayers. However, the influence of SM is less in the natural plasma membrane, perhaps because of modulating factors such as membrane proteins and the presence of a complex phospholipid mixture.

Introduction

Cellular cholesterol homeostasis involves a balance of the processes whereby unesterified (free, FC) cholesterol is supplied to and removed from the cell. Low-density lipoprotein (LDL) delivers cholesterol to peripheral cells by a well-characterized receptor pathway [1]. Understanding how cholesterol is transported in blood and lymph from peripheral cells to the liver for degradation and excretion is important in developing a model for the reversal of atherosclerosis. The primary metabolic role of high-density lipoproteins (HDL) may be this 'reverse cholesterol transport' from peripheral tissues to the liver (for reviews, see Refs. 2–5).

The surface transfer of FC between HDL and most cells growing in culture is bidirectional (i.e., FC is taken up and released simultaneously); the process occurs without the direct input of metabolic energy and involves diffusion of cholesterol molecules through the aqueous phase separating the HDL particles and the plasma membrane (for a review, see Ref. 4). The transfer of FC between red blood cells (RBC) and lipoproteins is also consistent with this aqueous diffusion

Abbreviations: BSA, bovine serum albumin; FC, free (unesterified) cholesterol; HDL_3, high-density lipoprotein-3; k_e, rate constant for efflux; k_i, rate constant for influx; LDL, low-density lipoprotein; PC, phosphatidylcholine; RBC, red blood cell; SUV, small unilamellar vesicles; SM, sphingomyelin; $t_{1/2}$, halftime.

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mechanism [6]. The rate of removal of cholesterol from cells to a given extracellular acceptor is influenced by the structure of the plasma membrane. Bellini and colleagues [7] demonstrated that the halftimes ($t_{1/2}$) for FC efflux from two types of rat liver cells were about 3 and 14 h. The same $t_{1/2}$ were observed for plasma membrane vesicles isolated from the cells. This suggests that the structures of the plasma membranes of the two cell types are different and that these differences account for differences in cholesterol efflux from the cells. To further understand the effect of plasma membrane structure on cholesterol efflux, we have used red blood cells. The use of RBC to investigate FC flux has several advantages: (1) the RBC membrane is well-defined and well-characterized, (2) the RBC is essentially an isolated plasma membrane which contains neither lipoprotein receptors nor intracellular organelles, (3) there is neither de novo synthesis of cholesterol nor esterification of cholesterol in RBC, (4) RBC from different species have different lipid compositions, and (5) the flux of FC between RBC and lipoproteins is physiologically significant because it is an integral part of the transport of cholesterol through the plasma compartment.

The exchange of cholesterol between RBC and plasma has been studied extensively since 1951 when the phenomenon was first established [8]. Most investigators report the presence of one pool of cholesterol in the human RBC [9–11]. The reported $t_{1/2}$ for cholesterol efflux from human RBC ranges from 2 to 8.2 h under conditions where desorption of cholesterol molecules from the plasma membrane is apparently rate-limiting (for a review, see Ref. 4). To investigate the effects of membrane lipid composition on the rate of FC desorption from the plasma membrane, we compared cholesterol efflux from bovine, rat, and rabbit RBC to efflux from human RBC. The dependence of FC exchange rate on the relative contents of sphingomyelin and phosphatidylcholine in the RBC membranes was evaluated and compared to the effect in a model membrane system comprised of small unilamellar vesicles.

Materials and Methods

Materials

Sources of chemicals were as follows: [4-^{14}C]cholesterol (50 mCi/mmol), Research Products International Corp. (Mount Prospect, IL); [7-^{3}H]cholesterol (12 Ci/mmol), ICN Biomedicals, Inc. (Costa Mesa, CA); [1,2-^{3}H]cholesterol (40–60 Ci/mmol), New England Nuclear, (Boston, MA); *N*-ethylmaleimide and bovine serum albumin (BSA; Fraction V, fatty acid-free), Sigma (St. Louis, MO); media and antibiotics, GIBCO (Grand Island, NY); sera for media, Flow Laboratories, Inc. (McLean, VA). Egg phosphatidylcholine and egg sphingomyelin were supplied by Calbiochem-Behringer Corp. (La Jolla, CA); the purities of these materials were assessed as described previously [12]. All other chemicals were of reagent grade quality.

Solutions used routinely in these studies were: buffer 1: Eagles' minimum essential medium buffered to pH 7.4 with 14 mM Hepes, and 0.05 mg of gentamicin/ml to prevent bacterial contamination; buffer 1 + 0.2% (w/v) BSA; buffer 2: 0.15 M NaCl, 10 mM Hepes (pH 7.0); buffer 2 + 0.2% (w/v) BSA.

Methods

Preparation of lipoproteins. Human LDL ($1.019 < d < 1.066$ g/ml) and HDL_3 ($1.125 < d < 1.21$ g/ml) were isolated by sequential ultracentrifugation [13]. HDL_3 was either labeled with [^{3}H]cholesterol as described by Johnson et al. [13] to a specific activity of about 1155–2000 cpm/μg FC or used without a label. [^{3}H]Cholesterol was purified by TLC prior to labeling HDL_3. Apolipoprotein integrity was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [14]. HDL_3 was dialyzed against buffer 1 for 24 h immediately before use. LDL was dialyzed into 0.15 M NaCl, concentrated about 20-fold, and either used directly for experiments or labeled with [^{3}H]cholesterol as described for HDL_3 to a specific activity of 600–685 cpm/μg FC.

Analytical procedures. HDL_3 and LDL lipids were extracted according to Bligh and Dyer [15]. RBC lipids were extracted by a modification of the method of Lange and colleagues [9] described below. Free and total cholesterol were quantitated by gas-liquid chromatography, using coprostanol as an internal standard [16]. Lipid phosphorus was determined by the method of Rouser et al. [17]. Protein was determined by the Lowry procedure, as modified by Markwell et al. [18], using bovine serum albumin as a standard. Radioactivity was measured by scintillation counting in a Beckman LS6800 counter using standard dual-label procedures to determine counts due to a specific isotope. SM and PC were determined by thin-layer chromatography (chloroform/methanol/water, 65 : 25 : 4, v/v) on 250 μm Anasil G plates from Analabs (North Haven, CT). The SM and PC were visualized by I_2 staining and were then scraped and the phosphorus determined as described above.

Preparation of cells. (A) Human: fresh blood was collected in 3.5 mM EDTA from healthy male donors. RBC were separated from plasma by centrifugation, washed three times in buffer 1, and then stored at 4° C. (B) Bovine: blood from Holstein cows was collected in EDTA and prepared as described above. (C) Rabbit: blood from male New Zealand White rabbits was collected in EDTA and prepared as described above. (D) Rat: blood from Lewis rats was collected in EDTA and prepared as described above.

Labeling of RBC with [4-^{14}C]cholesterol. 2 ml of RBC (50% hematocrit) were added to 14 ml buffer 1 which

contained 5% fetal bovine serum, 0.1–0.3 μCi/ml [^{14}C]cholesterol and 0.1% (v/v) ethanol, and incubated at 37°C for 4–6 h. The mixture was washed two times with buffer 1 and once with buffer 1 + 0.2% BSA. The cells were resuspended to a 50% hematocrit in the same buffer, stored at 4°C, and used within 8 days of initial isolation. This labeling procedure gave a specific activity of 1700–3200 cpm/μg RBC FC.

Assay of cholesterol exchange between RBC and lipoproteins. [^{14}C]Cholesterol-labeled human RBC were added to buffer 1 + 0.2% BSA to a final hematocrit of 4–8%. We utilized a low hematocrit although the physiological hematocrit of human RBC is about 42% because it is difficult to keep this concentration of RBC suspended in solution and well mixed. In addition, at the physiological hematocrit, extremely large quantities of lipoproteins are required to give a sufficient excess of lipoprotein cholesterol to RBC cholesterol. HDL_3 (or LDL) was added at the indicated concentration and 100 μl aliquots of this mixture were added to 1.5 ml Eppendorf tubes. The aliquots were incubated at 37°C in a warm box for the indicated times, shaken at 300 rpm by an orbital shaker. Samples were assayed by a modification of the procedure of Lange et al. [9] which is described briefly here. After the incubation, the Eppendorf tubes were placed on ice for 1 min. The incubation mixture in the Eppendorf tube was then subjected to centrifugation for 1 min at 12000 × *g* in a Beckman Microfuge. The supernatant was carefully removed, 1 ml of buffer 2 + 0.2% BSA was added to the cell pellet and this mixture was vortexed to resuspend the RBC. The aliquots were subjected to centrifugation and then washed a second time with buffer 2. The washed cell pellet was resuspended and extracted with 1 ml isopropanol for 30 min at room temperature. The mixture was subjected to centrifugation, and the resulting isopropanol cell extract was dried under N_2 and aliquots were taken for lipid and isotopic analyses. Lipids were extracted from the supernatant for analysis [15]. Bovine RBC were treated as above; rat and rabbit RBC were treated as above except that the aliquots were subjected to centrifugation at 5000 × *g* and 3000 × *g*, respectively. Except for the data in Fig. 2, three different preparations of RBC were used to determine each of the reported rate constants for cholesterol flux.

Control incubations demonstrated that both the association of HDL_3 FC with the plastic Eppendorf tube and the release of cellular cholesterol to media lacking HDL_3 were negligible; similar results were obtained with LDL. Control incubations were conducted in each experiment to correct for non-specific binding of lipoprotein to the RBC. The fraction of [^{3}H] isotope bound to RBC at 0 h was subtracted from each subsequent timepoint; for [^{3}H]HDL_3, this value was in the range 0.06–0.12. The fraction of [^{3}H]LDL that bound to RBC at 0 h ranged from 0.06 to 0.23. This high degree of association of LDL with RBC made it difficult to measure k_i for LDL FC reliably. Due to incomplete separation of RBC, a small fraction of [^{14}C]isotope remained in the supernatant at 0 h; this value of 0.01–0.06 was subtracted from each subsequent timepoint. Hemolysis was determined as described [9] and was less than 5% for all experiments.

Thin layer chromatography analysis of the [7-^{3}H]cholesterol used in some of our experiments showed it to be apparently > 98% pure cholesterol, but its behavior as a substrate for cholesterol oxidase revealed that a significant fraction of the label was an unidentified sterol closely related to cholesterol [19]. Impurities in apparently chromatographically pure tritiated cholesterol have been reported previously [20,21]. However, when RBC were double-labeled with [^{14}C]cholesterol and either [7-^{3}H]- or [1,2-^{3}H]cholesterol (which is > 99% cholesterol by TLC and cholesterol oxidase analysis), efflux of each isotopically-labeled cholesterol was similar. These results indicate that despite the impurity in the [7-^{3}H]cholesterol, the $t_{1/2}$ for its efflux are within the range observed for the two other isotopes.

Assay of cholesterol exchange between small unilamellar vesicles (SUV). The SUV were prepared and the cholesterol exchange kinetics were determined using procedures described previously [12]. Briefly, negatively charged donor phospholipid vesicles composed of 10 mol% [^{14}C]cholesterol, 15 mol% dicetyl phosphate and 75 mol% phospholipid were incubated with a 10-fold excess of acceptor vesicles (10 mol% cholesterol, 90 mol% egg PC and a trace of [^{3}H]cholesteryl oleate). The phospholipid in the donor SUV was egg PC and egg SM mixed in the indicated weight ratios. The donor and acceptor SUV were incubated at 45°C which is above the gel-liquid crystal transition temperatures of the phospholipids. The neutral acceptor vesicles were separated from the negatively charged donor vesicles on short columns of DEAE-Sepharose as described previously [22].

Analysis of kinetics of cholesterol flux. The uptake and release of FC were determined, respectively, from the accumulation of ^{3}H radioactivity in cells and the release of ^{14}C radioactivity to the media. The kinetics of tracer movement were analyzed using procedures described by Johnson et al. [13]. Briefly, two pools of FC were assumed; one comprised of all of the RBC FC and the other comprised of all of the HDL_3 FC (or LDL FC), and together these two pools of FC formed a closed system. Computer fitting of the experimental time courses of tracer uptake and release to the above kinetic model yielded the first-order rate constants of FC influx (k_i) and efflux (k_e).

The values of k_i presented here in terms of h^{-1} are dependent on the volume of medium applied to cells and the number of cells exposed to this HDL-containing medium. To remove the dependence on medium

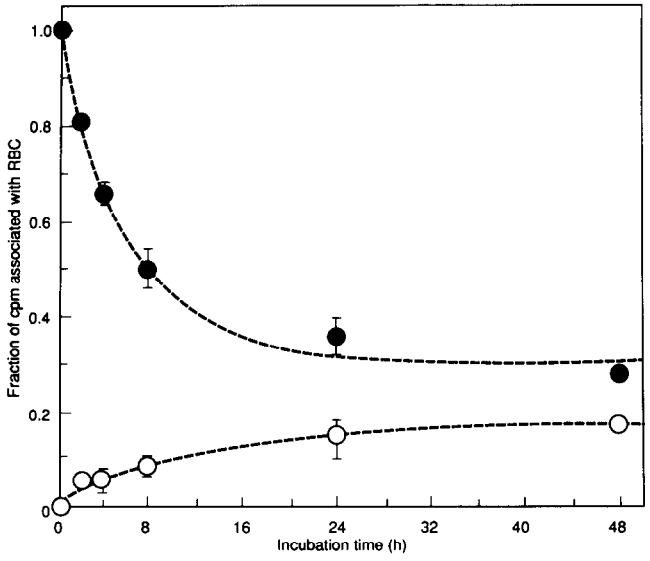

Fig. 1. Time courses of the bidirectional efflux of cholesterol between human HDL_3 and human RBC. Suspensions of 5–10% hematocrits of RBC (6.1–7.2 μg cholesterol, 1745–3300 cpm [^{14}C]FC/μg FC) were incubated with HDL_3 (0.15 μg HDL_3 FC/μl, 1155–1990 cpm [^{3}H]FC/μg FC) in a final volume of 100 μl of MEM-Hepes containing 0.2% BSA. Aliquots were incubated with gentle shaking at 37 °C for the indicated times, whereupon the cells and medium were then separated and analyzed. Results are expressed as the fraction of [^{3}H]FC originally in medium transferred to cells (○------○) or the fraction of [^{14}C]FC originally in cells remaining in cells (●------●) versus incubation time. Each point is the mean (±S.D.) of $N = 9$ determinations. The dashed lines were obtained by computerized fitting of the experimental data to a model assuming that cell-associated and lipoprotein-associated FC each form a single homogeneous pool and that together the pools form a closed system (see Methods).

volume and cell number, and to allow comparisons between different cell types, or laboratories, values of k_i may be re-expressed in terms of clearance of HDL FC by cells. CL_i is equal to $k_i \times$ volume of HDL solution applied to cells/number of cells [13].

Statistical comparisons were performed by one-way anova and post-hoc treatment of the data by Tukey's test [23].

Results and Discussion

Cholesterol flux between human RBC and human HDL_3 and LDL

The studies reported here are the first bidirectional flux studies which measure directly the rate constants for both influx and efflux of cholesterol from the red blood cell to HDL_3 or LDL. A typical time course for cholesterol exchange between human RBC and HDL_3 is shown in Fig. 1. The experimental data fit the theoretical curves (dotted lines), based on the kinetic model with one pool of cholesterol in the RBC and one in the HDL_3. The efflux data are linear when plotted in semi-exponential form as the fraction of counts remaining in the cell vs. time, as expected for a first-order process (data not shown). The values of the rate constants of cellular FC influx and efflux derived from the computer analysis of the kinetics of FC exchange are listed in Table I. The agreement between theory and experiment demonstrates that cholesterol in human RBC behaves kinetically as a single, fully exchangeable pool of cholesterol which agrees with previous work on lipoproteins and RBC (cf. Refs. 9–11) as well as work on RBC and vesicles (cf. Refs. 24–26). The recent study by Brasaemle et al. [27] reports that the halftime for translocation of cholesterol between the outer and inner phospholipid monolayers of the membrane is 50–130 min. This indicates that the rate of cholesterol translocation is 3–5-fold faster than the rate of transfer from the membrane, and would not be the rate-limiting step.

The redistribution of tracers in our experiments could be followed for 48 h with 2% or less hemolysis of the RBC, but the exchange of cholesterol was essentially complete after 24 h. The masses of cholesterol in both the RBC and the lipoprotein were measured at 0 and 24 h and did not change confirming that there was no net transfer of FC mass. Since all of the cholesterol in the RBC membrane and HDL_3 is exchangeable (Fig. 1 and Refs. 9–11, 24–26), the specific activities of the two isotopic tracers should coincide at equilibrium. For the data in Fig. 1, the [^{14}C]cholesterol reaches the equilibrium value expected on the basis of the relative masses of cell and lipoprotein cholesterol. However, the [7-^{3}H]cholesterol fails by 10–20% to reach the expected equilibrium values. This lack of complete equilibration is peculiar to the [7-^{3}H]cholesterol and presumably reflects a contaminant in it (see Methods). When HDL_3 is labeled with [1,2-^{3}H]cholesterol, the cholesterol radioactivity does reach the predicted equilibrium value.

The rate constants for cholesterol influx to the RBC are also shown in Table I. At low and high HDL_3 concentrations, k_i equals 0.01 and 0.02 h^{-1}, respectively. This is within the range calculated by Gottlieb [11]. These rates have been converted to Cl_i, to remove the dependence on medium volume and cell number as discussed in Methods. Influx data are difficult to obtain with LDL because there are non-specific binding sites on RBC for LDL [28], which gives rise to a high background count of radioactively labeled LDL bound to the RBC. Even using low levels of labeled LDL, the values obtained for influx of cholesterol are very low (Table I); these values are reproducible, although only just above background values. The lower k_i for FC flux to RBC from LDL than from HDL_3 is expected because the minimal $t_{1/2}$ values for transfer of FC out of the two lipoproteins are 45 and 3 min, respectively [29].

The dependences on lipoprotein concentration of the rate constants for efflux of FC from RBC are depicted in Fig. 2. There is a hyperbolic dependence of k_e on the ratio of HDL cholesterol to RBC cholesterol with a maximal value of 0.10 h^{-1} (equivalent to $t_{1/2} = \ln 2/k_e$

TABLE I

Rate constants describing the bidirectional flux of cholesterol between human RBC and lipoproteins

The time courses were run under the conditions described in Fig. 1, with the indicated concentration of [^{3}H]FC lipoprotein and [^{14}C]FC human RBC. LDL specific activity was 600–685 cpm [^{3}H]FC/μg FC. The rate constants were determined by computer modeling, as described in Methods. Each measurement is the mean (±S.D.) of nine determinations.

Lipoprotein		Rate constant		$t_{1/2}$ for efflux of RBC FC (h)
Type	Concn.[a] (μg FC/μl)	k_i (h^{-1})	k_e (h^{-1})	
HDL_3	0.15	0.01[b]	0.12	6.0±0.8
HDL_3	0.72	0.02[c]	0.15	4.6±0.6
LDL	0.15	0.0002	0.07	9.7±1.9
LDL	2.6	–	0.11	6.2±0.2

[a] The lower concentration of lipoprotein is near the physiological (plasma) ratio of lipoprotein FC/RBC FC (assuming a physiological hematocrit of 42%); the higher concentration gives the minimal $t_{1/2}$.

[b] Clearance is $(1–2)\cdot10^{-9}$ μl/h per RBC. The cell number was estimated by assuming that at a 100% hematocrit there are about $9\cdot10^9$ RBC/ml [9].

[c] Clearance is $(3–4)\cdot10^{-9}$ μl/h per RBC.

= 6.9 h) reached at about 6-fold excess HDL cholesterol (over RBC cholesterol). When a number of time courses were performed at an excess HDL_3 concentration, this minimal $t_{1/2}$ was 4.6 ± 0.6 h (Table I). The difference between the two values is probably explained by the fact that the results in Fig. 2 are based only on a 6 h timepoint. A similar curve for LDL concentration-dependent efflux of FC from RBC is shown in Fig. 2B. At high levels of LDL, cholesterol efflux is essentially independent of LDL concentration and the fractional rate is 0.08 h^{-1} ($t_{1/2}$ = 8.2 h). This systematic study of cholesterol efflux from RBC to lipoprotein particles, employing a 600-fold range of acceptor concentrations confirms and extends prior work [10,11,26]. The observation that at high concentrations of lipoprotein acceptors, k_e is essentially independent of the concentration of acceptor particles (Fig. 2) agrees with results for FC exchange between SUV and is consistent with the aqueous diffusion mechanism for the RBC FC exchange process (reviewed in Ref. 4). At high extracellular concentrations of HDL_3 or LDL, the rate-limiting step of cholesterol efflux is desorption of cholesterol from the RBC membrane. The somewhat lower minimal $t_{1/2}$ of 4.6 h observed with HDL_3 (compared to 6.2 h with LDL) may be due to the transfer of some exchangeable apolipoproteins from HDL (apolipoprotein A-I, A-II or C) to the RBC surface, while apolipoprotein B from LDL is not transferable. Such a transfer of apolipoprotein has been observed to increase the rate of exchange from mitochondrial membranes [30] and egg PC SUV (Letizia, J.Y. and Phillips, M.C., unpublished data). The adsorption of apoprotein to the membrane presumably disrupts the FC–phospholipid interactions and facilitates desorption of cholesterol molecules.

It is apparent from Figs. 2A and 2B that k_e attains its maximal values when the ratios of lipoprotein to RBC FC masses are about 6 : 1 and 15 : 1 for HDL_3 and LDL, respectively. At these FC ratios, the respective particle concentrations of HDL_3 and LDL are $43\cdot10^{18}$ and $3\cdot10^{18}$ particles/L; this calculation is based on the lipoprotein compositions reported in Shen et al. [31]. The collision frequency (Z_{ca}) per unit volume of medium between cholesterol molecules which have desorbed from the cell and acceptor particles has the following dependence on acceptor particle size [32]: $Z_{ca} \propto R_a \cdot C_a$, where R_a = the hydrodynamic radius of the acceptor particle and C_a = particle concentration of acceptor. Since R_{HDL}

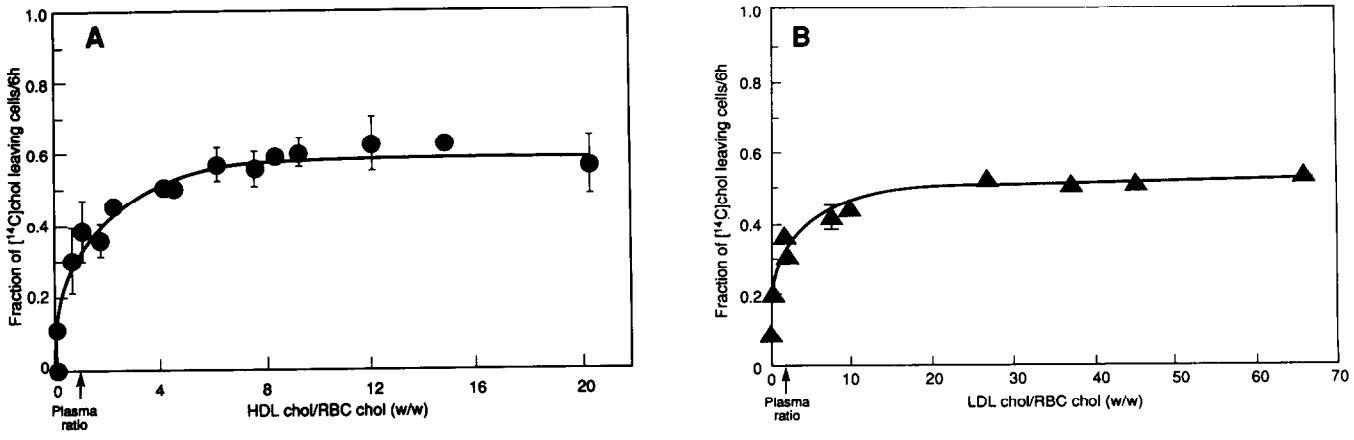

Fig. 2. Effect of lipoprotein acceptor concentration on the initial efflux of FC from human RBC. Incubation conditions and analyses were as described for Fig. 1, except that the incubations were for 6 h. Each point is the mean of six determinations in two separate experiments and error bars are included (±S.D.). (A) ●, Human HDL_3. (B) ▲, Human LDL. The vertical arrows indicate the physiological ratios of lipoprotein cholesterol to RBC cholesterol.

is about 3.9 nm and R_{LDL} is about 9.6 nm [31], it follows that under the above conditions the ratio $Z_{cHDL}/Z_{cLDL} = 5.9:1$. Therefore, to attain the plateau k_e values in Figs. 2A and 2B, 5.9-times more collisions between lipoprotein particles and desorbed cholesterol molecules are needed with HDL than with LDL. Since the same number of effective collisions (i.e., collisions that result in absorption of a cholesterol molecule into the acceptor lipoprotein particle) should be required to achieve the maximum k_e, the probability of a collision being effective is about 6-times greater for LDL than for HDL_3. This factor may be partially explained by the fact that a greater fraction of the LDL surface is covered by phospholipid rather than by apoprotein; the phospholipid/apoprotein w/w ratios for LDL and HDL_3 are about 1 and 0.5, respectively [31]. It should be noted that when the physiological ratios of HDL_3 and LDL FC to RBC FC are present (Figs. 2A and 2B), k_e is greater for HDL_3 than for LDL.

The kinetics described above are consistent with the aqueous diffusion mechanism where FC desorbs from the RBC membrane and diffuses through the aqueous phase to an acceptor particle [4,7,9]. In this model, the cholesterol molecules which have a small but finite solubility in water desorb from the RBC membrane and diffuse through the thick unstirred water layer around the cell [33] until they collide with and are absorbed by an acceptor particle. At low concentrations of extracellular particles, this absorption step can be rate-limiting for the overall efflux process. In contrast, at high acceptor concentrations, the rate-limiting step is the desorption of FC molecules from the cell plasma membrane. There is general agreement that the desorption of hydrophobic molecules such as cholesterol from the donor lipid/water interface proceeds through a transition-state in which the partially desorbed molecule is attached to the donor by the tip of its hydrophobic tail. The oscillatory motions of the FC molecule perpendicular to the lipid/water interface cause desorption when the activation energy is overcome. A variation of this model has been proposed [34]; in this case the partially desorbed cholesterol molecule is induced to transfer to an acceptor particle by a collision with the acceptor particle. The implication is that direct contact between the RBC membrane and acceptor lipoprotein particles is required. This model is not consistent with the results from several laboratories showing that cholesterol molecules can diffuse between donor and acceptor particles which are physically separated by either a dialysis membrane or a polymer phase [22,35,36].

Influence of relative SM/PC contents on cholesterol exchange kinetics in red blood cells and vesicle systems

It is well established from studies with model membranes that the lipid composition of the donor lipid/water interface can have a large effect on the rate of

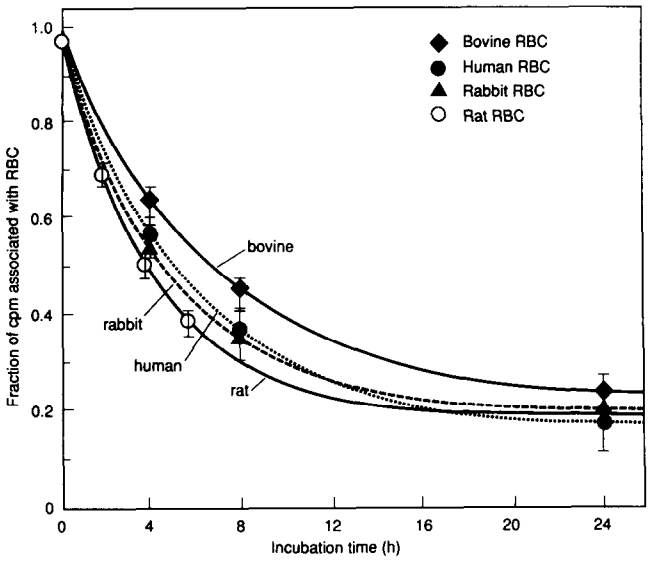

Fig. 3. Cholesterol efflux from four species of RBC to human HDL_3. Incubations and conditions were as described for Fig. 1 except that 6.7–11.8 μg RBC FC was incubated with a fixed concentration of HDL_3 (0.72 μg HDL_3 FC/μl). The specific activity of the RBC cholesterol was 760–3190 cpm [^{14}C]FC/μg FC. Each point is the mean (±S.D.) of nine determinations. ●, Human RBC; ◆, bovine RBC; ▲, rabbit RBC; ○, rat RBC. The lines were obtained by the computerized fitting procedure, as described for Fig. 1.

cholesterol transfer (for a review, see Ref. 4). It is known that the SM/PC ratio affects the kinetics of FC exchange from phospholipid/cholesterol bilayers and here we compare the effects of this parameter on cholesterol efflux from vesicles and RBC. Cholesterol efflux from four types of mammalian RBC with different levels of SM was determined and compared to the equivalent kinetics in a model membrane system comprised of small unilamellar vesicles formed by mixing different ratios of SM to PC.

Fig. 3 shows the time courses of cholesterol efflux from the four types of RBC (rat, rabbit, human and bovine) to excess HDL_3. The time courses are similar for all RBC except that cholesterol leaves the bovine cells a little more slowly than from the other types of cells. Table II summarizes the $t_{1/2}$ for the efflux of cholesterol for each species; the $t_{1/2}$ values for rat and

TABLE II

Cholesterol efflux from RBC from different mammals

Type of RBC	SM content (% of total PL) (mol/mol)	SM/PC (w/w)	Halftime for FC efflux (h) [a]
Rat	19	0.3/1	3.7±0.6 [c]
Rabbit	20 [b]	0.6/1 [b]	4.0±0.2 [c]
Human	25	0.9/1	4.6±0.6
Bovine	46	5.0/1	5.5±0.8

[a] See Fig. 3 for incubation conditions.
[b] This value was obtained from Ref. 38.
[c] This valuc is significantly different from the $t_{1/2}$ for bovine RBC ($P < 0.05$, $N = 9$).

TABLE III

Kinetics of cholesterol exchange from egg PC/egg SM vesicles

mol% SM	Halftime (h) [a] at 45 °C
0	0.3 ± 0.1
25	1.1 ± 0.0
50	3.4 ± 0.3
75	6.9 ± 1.5
100	17.0 ± 2.0

[a] The $t_{1/2}$ values are mean values (±S.E.) from five kinetic experiments using two preparations of donor vesicles.

rabbit cells are significantly shorter than the $t_{1/2}$ for bovine RBC. These results agree with previous data [37] which suggested that there is no simple correlation between the rate of cholesterol exchange and the SM content of rat, human and sheep RBC. The relative SM contents of the RBC are also listed in Table II. The principal difference in phospholipid composition between the four species of RBC is the ratio of SM/PC (Table II and Ref. 38). When the acyl chains of the PC in a given species of RBC are relatively saturated (i.e., the unsaturated/saturated chain ratio is low), the SM molecules also tend to be saturated. For instance, rat and bovine PC have unsaturated/saturated chain ratios of about 0.5 and the equivalent value for the SM of both species is in the range of 0.2–0.35 [38]. Rabbit and human RBC contain PC and SM with unsaturated/saturated chain ratios of about 1.1 and 0.8, respectively [38]. Consequently, the variations in SM/PC ratio between the four species of RBC are not reflected in major changes in the degree of acyl chain unsaturation.

Table III shows how changing the ratio of egg SM to egg PC influences cholesterol exchange in small unilamellar vesicles. It is apparent that as the ratio of SM/PC increases, the $t_{1/2}$ for cholesterol exchange increases: raising the SM content from 25 to 75% increases the $t_{1/2}$ from about 1 h to 7 h. Since the experiments in Table III were conducted at 45 °C where the bilayers are in a liquid-crystalline state (the transition temperature of egg SM is about 42 °C), the increase in $t_{1/2}$ reflects the slower desorption of cholesterol molecules as the SM content of the liquid-crystal donor membrane is increased. Because, relative to the equivalent PC, SM molecules adopt a more condensed lateral packing in bilayers and interact more strongly with cholesterol, the Van der Waals interactions of cholesterol with the host phospholipid are stronger and the rate of cholesterol desorption is slower [12]. Similar increases in $t_{1/2}$ as the ratio of SM/PC increases have been reported in other model membrane systems (see Refs. 12,39–42). Similarly, when the SM content of *Mycoplasma gallisepticum* membranes was increased from 0 to about 50%, the $t_{1/2}$ for cholesterol efflux increased from 1.1 h to 4.7 h [43].

When the halftimes for efflux of cholesterol from the four species of RBC (Table II) and from the egg PC/egg SM vesicles (Table III) are replotted together as a function of the mol% SM in the donor membrane (Fig. 4), it is possible to compare directly the influence of SM content in a model and a natural membrane. Qualitatively, $t_{1/2}$ increases with increasing SM content in both systems. However, the influence of increasing the SM level is greater in the egg PC/egg SM vesicle system. For example, raising SM from about 20 to 50% is associated with a 3-fold increase in $t_{1/2}$ from about 1 h to 3 h while in RBC $t_{1/2}$ increases by a factor of only about 1.5 (3.7 h for rat RBC to 5.5 h for bovine RBC). Factors which contribute to the quantitative differences between SUV and RBC probably include the differences in FC content (10 mol% in SUV and about 40 mol% in RBC) and the complex acyl chain composition of RBC compared to that of the egg PC/egg SM mixed bilayer. Also, the fact that the cholesterol exchange from RBC was performed at 37 °C while that from egg PC/egg SM SUV was performed at 45 °C contributes to the shorter $t_{1/2}$ observed with the SUV. Finally, the composition of the outer leaflet of the membrane is presumed to have the major effect on the rate of cholesterol desorption into the extracellular aqueous phase. The mol% SM plotted on the abscissa of Fig. 4 reflects the total phospholipid composition of the membrane and would only reflect the composition of the outer monolayer if the transbilayer phospholipid distribution were symmetrical. In fact, the SM is preferentially located in the outer monolayer (80% of the SM is in this location in human RBC [44]) so the SM content of the outer monolayer of RBC is underrepresented on the abscissa of Fig. 4.

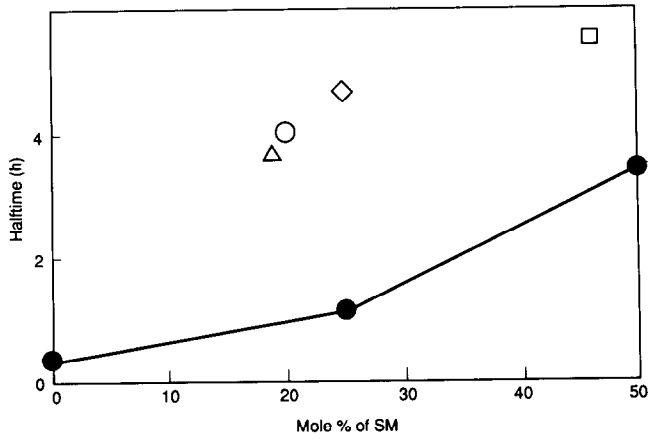

Fig. 4. The influence of the SM content of RBC and egg PC unilamellar vesicles on the kinetics of cholesterol efflux. The mol% SM plotted on the abscissa is relative to the total phospholipid composition of the membrane. $t_{1/2}$ for transfer of [^{14}C]cholesterol from egg PC/egg SM vesicles at 45 °C, ●———●; $t_{1/2}$ for efflux of cholesterol from different species of RBC at 37 °C, ○, rabbit; △, rat; ◇, human; □, cow.

The results described in this paper suggest that increasing the membrane SM/PC ratio raises the $t_{1/2}$ for cholesterol exchange in both the RBC plasma membrane and in simple mixed SM/PC bilayers. However, the influence of SM is less in the natural plasma membrane, perhaps because of modulating factors such as membrane proteins and the presence of a complex phospholipid mixture. More work is required to elucidate the roles of the various plasma membrane components in cholesterol efflux.

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