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Effects of membrane lipids and -proteins and cytoskeletal proteins on the kinetics of cholesterol exchange between high density lipoprotein and human red blood cells, ghosts and microvesicles

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To better understand the effects of plasma membrane lipids and proteins and the cytoskeleton on the kinetics of cellular cholesterol efflux, the effects of (1), selectively depleting either sphingomyelin (SM) or phosphatidylcholine (PC); (2), cross-linking the cytoskeleton, and (3), removing certain cytoskeletal and integral membrane proteins on radiolabelled cholesterol efflux from red blood cells (RBC) have been studied. When RBC were treated with either phospholipase A_2 or sphingomyelinase C to hydrolyze either 30–40% of the PC or 40–50% of the SM, respectively, the halftimes ($t_{1/2}$) for cholesterol efflux to excess HDL_3 were not significantly altered, with the values being 4.4 ± 0.8 h or 3.7 ± 0.4 h, respectively, compared to 4.6 ± 0.6 h for control RBC. To investigate the effects of the cytoskeleton on the rate of free cholesterol (FC) desorption from the plasma membrane, the cytoskeletal proteins were cross-linked by either heat-treatment or exposure to diamide and cholesterol efflux from ghosts of these cells was measured. Cross-linking the cytoskeletal proteins by diamide treatment resulted in no significant change in $t_{1/2}$ for treated (3.6 ± 0.6 h) compared to control (4.2 ± 0.4 h) ghosts; this suggests that the cytoskeleton does not play a large role in modulating cholesterol efflux. To investigate the effects of membrane proteins on cholesterol efflux, RBC microvesicles, containing mainly band 3 and 4 proteins and little of the cytoskeletal proteins, such as spectrin (bands 1,2) or actin (band 5), were obtained by incubation with the ionophore A23187. With excess HDL_3 present, microvesicles exhibited a $t_{1/2}$ of 4.2 ± 1.9 h (compared to the $t_{1/2}$ of 4.2 ± 0.4 h for control ghosts). The results described in this paper suggest that neither changing the SM/PC ratio in the membrane nor cross-linking the cytoskeletal proteins nor removing the cytoskeleton changes the $t_{1/2}$ for cholesterol efflux to excess HDL_3. Presumably, the cholesterol-phospholipid interactions are insensitive to these perturbations in membrane structure.

Introduction

The structure of the red blood cell (RBC) membrane and cytoskeleton has been studied in great detail. Consequently, the RBC system affords opportunities not possible with other cell types to examine directly the effects of membrane organization on cholesterol efflux to extracellular lipoprotein particles [1–4]. For example, it is possible to obtain 'cytoskeleton-free' preparations of membranes and to cross-link proteins and examine efflux from ghosts when the treated cells are too fragile to withstand cholesterol efflux without hemolysis. It is known that the plasma membrane structure is critical in determining the halftime ($t_{1/2}$) for cellular cholesterol efflux. Thus, Bellini et al. [5] demonstrated that two types of rat liver cells had different $t_{1/2}$ for cholesterol efflux and that plasma membrane vesicles isolated from the two types of cells gave $t_{1/2}$ values identical to the parent cells.

Previously, we have investigated the effects of membrane-lipid composition on the rate of cholesterol (FC) desorption from the plasma membrane by comparing cholesterol efflux from bovine, rat and rabbit RBC to efflux from human RBC [4]. By using established tech-

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Abbreviations: BSA, bovine serum albumin; FC, free (unesterified) cholesterol; HDL, high density lipoprotein; k_e, rate constant for efflux; PC, phosphatidylcholine; PL, phospholipid; PLA_2, phospholipase A_2; RBC, red blood cell; SM, sphingomyelin; SMase C, sphingomyelinase C; $t_{1/2}$, halftime; TLC, thin-layer chromatography.

niques, we manipulate either lipid composition or cytoskeletal and membrane protein organization and examine the effects of these structural determinants on cholesterol efflux from the human RBC membrane.

Materials and Methods

Materials

Sources of chemicals were as follows: [4-^{14}C]cholesterol (50 mCi/mmol), Research Products International (Mount Prospect, IL); [1,2-^{3}H]cholesterol (40–60 Ci/mmol), New England Nuclear (Boston, MA); Phospholipase A_2 (PLA_2) from *Naja naja* and bee venom, sphingomyelinase C (SMase C) from *Staphylococcus aureus*, ionophore A23187, bovine serum albumin, fraction V (fatty acid-free), diamide, merocyanine 540, Sigma (St. Louis, MO); media and antibiotics, Gibco (Grand Island, NY); Millipore ultrafree MC filter units (0.2 μm), Millipore (Bedford, MA); Sepharose CL-4B, PhastGel gradient 4–15% gel, PhastGel SDS Buffer Strips, Pharmacia (Piscataway, NJ).

Methods

Buffers. 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/ml of gentamycin to prevent bacterial contamination; buffer 2: buffer 1 + 0.2% (w/v) BSA; buffer 3: 0.15 M NaCl + 1 mM EDTA; buffer 4: 90 mM KCl, 45 mM NaCl, 44 mM sucrose, 30 mM Hepes (pH 7.4); buffer 5: 0.154 M NaCl, 10 mM Hepes (pH 7.4); buffer 5 + 4 mM $CaCl_2$ + 2.2 μg/ml A23187 (in DMSO); buffer 5 + 20 μM merocyanine 540.

Analytical procedures. HDL_3 lipids were extracted according to Bligh and Dyer [6]. RBC lipids were extracted by a modification of the method of Lange and colleagues [1], as described previously [4]. Free cholesterol was quantitated by gas liquid chromatography, using coprostanol as an internal standard [7]. Lipid phosphorus was determined by the method of Rouser et al. [8]. Protein was determined by the Lowry procedure, as modified by Markwell et al. [9], using BSA 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. Sphingomyelin (SM), phosphatidylcholine (PC), phosphatidylethanolamine and lyso-PC were determined by thin-layer chromatography (TLC) (chloroform/methanol/water, 65:25:4, v/v) on 250-mm Anasil G plates from Analabs (North Haven, CT). The phospholipid bands were visualized by iodine staining, scraped and the phosphorus was determined as described above.

Preparation of lipoproteins. Human HDL_3 ($1.125 < d < 1.21$ g/ml) was isolated by sequential ultracentrifugation as described previously by Johnson et al. [10].

Preparation of cells. Human blood was collected as described previously [4] and labelled with 1,2-[^{14}C]cholesterol to a specific activity of 800–1500 cpm/μg cholesterol. Labelled RBC were stored in buffer 2 at 4°C and used within 7 days of collection.

Preparation of lipase-treated RBC. Cholesterol-labelled RBC were treated with 36 units of bee venom PLA_2 together with 40 U/ml cells of *Naja naja* PLA_2 in buffer 1 + 10 mM $CaCl_2$ for 60 min at 37°C [11,31]. The reaction was stopped by washing the RBC three times with saline + 1 mM EDTA + 0.2% BSA. The cells were washed once in buffer 2, resuspended to a 50% hematocrit and stored at 4°C (and used within 2 days of the treatment). The procedures of Verkleij et al. [11] were also used to treat RBC with sphingomyelinase C (SMase C). Labelled RBC were treated with 38 mU/ml cells of *Staphylococcus aureus* SMase C in buffer 1 for 20 min at 37°C. The reaction was stopped as described for the PLA_2-treated cells; the RBC were stored at 37°C (and used within 1 day of the treatment). The SMase C-treated cells were cold-labile [12].

The phospholipids from both types of lipase-treated cells were characterized by TLC and phosphorus assays to determine the changes in phospholipid content due to lipolysis.

Cholesterol exchange experiments. The kinetics of cholesterol exchange between RBC and extracellular HDL_3 were monitored as described [4]. Briefly, the labeled RBC (at a 5–10% hematocrit) were incubated with HDL_3 (at a 10-fold excess of acceptor HDL_3 in terms of cholesterol concentration) in buffer 2. The mixture was shaken at 37°C for the desired time before the cells and lipoprotein were separated by centrifugation. The supernatant containing the lipoprotein was analyzed directly by scintillation counting; the RBC pellet was washed twice and then the cholesterol was extracted and counted. The cholesterol efflux experiments with RBC ghosts were performed as described above for RBC, except that the ghosts and HDL_3 were separated by filtration through a 0.2 μm Millipore filter unit. Control experiments showed that the recovery of lipoprotein in the filtrate was > 95% and that no counts due to ghosts could be detected in the filtrate.

The fraction of [^{14}C]cholesterol in the lipoprotein was determined for each time-point in triplicate. The resulting time-course was fitted to a model for exchange between two homogeneous pools as described previously [4] using non-linear regression (Enzfitter, Elsevier Biosoft, Cambridge, UK). The rate constant (k_e) and the halftime ($t_{1/2}$) for cholesterol efflux from the RBC were derived from this analysis.

Preparation of RBC ghosts. Ghosts were prepared according to Steck et al. [13] from [^{14}C]cholesterol-labelled RBC, using 5 mM sodium phosphate (pH 7.6). The labelled ghosts were resealed by an incubation at

37°C for 40 min in phosphate-buffered saline and then washed three times in buffer 2.

The cholesterol and protein contents of the ghosts were determined (and were in the range of 0.15–0.25 mg chol/mg protein). The distribution of proteins was determined by SDS-PAGE by adapting the method of Steck and colleagues [14] to the Pharmacia PhastSystem; a 4–15% SDS gradient gel was used and the positions of the major protein bands were determined for ghosts and RBC.

Preparation of diamide-treated ghosts. Labelled RBC were cross-linked with diamide following the procedure described by Franck and colleagues [15]. Briefly, RBC were washed 3 times in buffer 3 and then incubated in buffer 3 + 5 mM diamide for 40 min at 37°C. The cells were then washed once in buffer 3 and twice in phosphate-buffered saline before ghosts were prepared as described above (cross-linked RBC were too fragile to conduct cholesterol exchange experiments, since the cells lysed within 30 min of incubation). Cross-linking of cytoskeletal proteins by diamide was verified by SDS-PAGE, as described above.

Preparation of heat-treated ghosts. Labelled RBC (10% hematocrit) were heated for 15 min at 50°C in buffer 1 [16]. The cells were washed three times with buffer 1 to remove microvesicles and then were prepared as ghosts, as described above. Cross-linking of cytoskeletal proteins was confirmed by gel electrophoresis.

Preparation of microvesicles. Microvesiculation of RBC was induced with the ionophore A23187 [17]. Cholesterol-labelled RBC were incubated with 1 volume of buffer 5 + 4 mM $CaCl_2$ + 2 μg/ml A23187 (in DMSO) for 16 hours at 37°C (at a 50% hematocrit). This mix was washed and the pellet resuspended in buffer 5 and centrifuged for 5 min at $500 \times g$ to remove RBC and then centrifuged twice for 30 min at $16000 \times g$ to wash the microvesicle pellet. The pellet was resuspended in buffer 5 and characterized by gel electrophoresis and analyzed for protein and cholesterol contents. The yield was about 1%. Microvesicles were also obtained by exposing RBC to merocyanine 540 [18]. Labelled RBC (at a 10% hematocrit) were incubated in buffer 5 + 20 μM merocyanine 540. The mixture was incubated for 15 min at 37°C and then centrifuged to remove the RBC. The supernatant was spun for 30 min at $16000 \times g$ to pellet the microvesicles. This pellet was resuspended in buffer 1 and characterized as described above for the A23187 microvesicles. The yield for merocyanine 540 microvesicles was extremely low (< 0.1%).

Cholesterol-exchange experiments with microvesicles and HDL_3. Cholesterol-labelled microvesicles were incubated with HDL_3 as described above for RBC exchange experiments. At each time-point, a 100 μl aliquot of the incubation mixture was applied to a 2 ml Sepharose CL-4B column equilibrated in buffer 2 (at room temperature). These columns had previously been calibrated using labelled microvesicles and [^{3}H]HDL_3. The fractions containing the lipoprotein were collected (in about 4 min) and counted by scintillation counting and analyzed as described above for the RBC system. Appropriate controls were run to determine the amount of contamination of HDL_3 with microvesicles (< 5%) and the recovery of HDL_3 from the columns (> 95%).

Results and Discussion

The aim of the studies presented here is to elucidate how various plasma-membrane components affect the rate of cholesterol release from cells. The influence of the following perturbations of membrane and cytoskeleton structure on the kinetics of cholesterol efflux from red blood cells, RBC ghosts and RBC microvesicles have been monitored. (1) The choline-containing phospholipids (phosphatidylcholine and sphingomyelin) were depleted by exposure to phospholipases. (2) The cytoskeletal proteins were cross-linked and immobilized by treatment with either diamide or heat. (3) The cytoskeleton was detached by the formation of microvesicles.

Membrane lipids

To investigate the roles of membrane lipids on cholesterol efflux, we focussed on two of the lipids found in the outer leaflet of the membrane of the RBC, phosphatidylcholine (PC) and sphingomyelin (SM). 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 cholesterol transfer (for a review, see Ref. 19). The rate of cholesterol exchange is much slower from model membranes with a high SM/PC ratio [20–24]; this occurs because of greater Van der Waals attraction between cholesterol and SM than cholesterol and PC [20,25]. Previous results from this laboratory [4] comparing cholesterol efflux from RBC of different mammalian species suggested that increasing the membrane SM/PC ratio slightly raises the $t_{1/2}$ for cholesterol exchange. In the work presented here, we focus exclusively on human RBC and use lipases to alter the PC or SM in the membrane to further examine the effects of these membrane components on cholesterol exchange.

Fig. 1 shows the time-courses of cholesterol efflux from control and SMase C-treated RBC. The lipase-treated cells show a similar halftime for cholesterol exchange to the untreated cells. Table I summarizes the $t_{1/2}$ for the efflux of cholesterol for control, PLA_2- and SMase-C-treated RBC. The PLA_2-treated cells have 30–40% of their PC removed and yet the $t_{1/2}$ is the same as for the untreated cells. With the SMase-

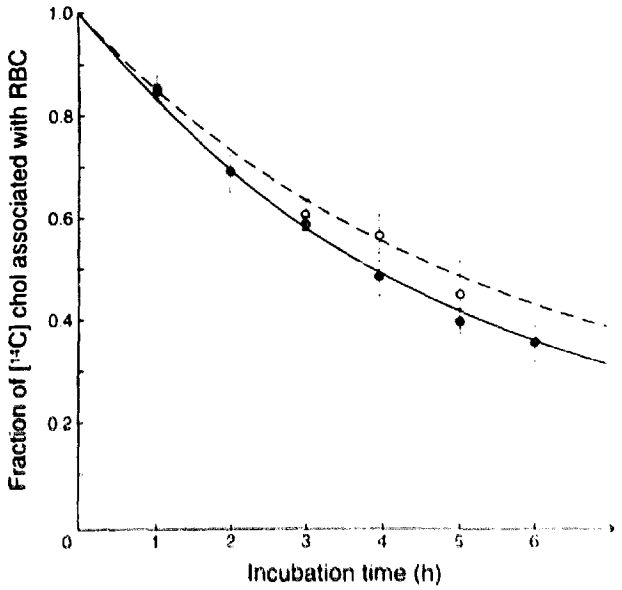

Fig. 1. Time-courses of cholesterol efflux from control and SMase C-treated human RBC to human HDL_3. RBC were treated with SMase C as described in Materials and Methods. Suspensions at 5–10% hematocrits of RBC (6.2–7.3 μg cholesterol, 810–1560 cpm [^{14}C]FC/μg FC) were incubated with HDL_3 (final concentration of 0.72 μg FC/μl) in a final volume of 100 μl of buffer 1 + 0.2% BSA. After incubation with gentle shaking at 37°C for the indicated times, the cells and medium were separated and analyzed. The fraction of [^{14}C]FC remaining in the cells was determined as a function of the incubation time. Each point is the mean (+S.D.) of nine determinations. The lines were obtained by computerized fitting of the experimental data as described in Materials and Methods. ○·····○, control RBC; ●——●, SMase C-treated RBC.

C-treated cells, 40–50% of their SM is converted to ceramide and the $t_{1/2}$ is slightly lower (3.7 h), although the difference is not statistically significant (by Tukey's test [26]). The minor effect of changing the SM and PC contents by lipases in the RBC membranes is consistent with previous work from this laboratory [4], where the $t_{1/2}$ for cholesterol efflux from four species of RBC with widely varying SM/PC ratios showed only a slight increase with increasing SM/PC ratio.

The effects on RBC of the two lipases used in the work presented here have been thoroughly described by various authors [11,27–31]. The changes in SM/PC ratio reported in Table I are consistent with the earlier work. Treatment of RBC with PLA_2 results in a shape-change from biconcave to echinocyte, detectable under a light microscope which agrees with previous results [27,28] and our data (not shown). Exposure of RBC to SMase C leads to the formation of stomatocytes [27,28]. The use of other lipases was precluded by the resultant lysis of the cells or by the fact that the appropriate PL was not in the outer half of the plasma membrane and, thus, not available to the lipase [11]. The action of lipases on RBC ghosts is much less specific than in whole cells, perhaps due to the 'leakiness' of the ghosts. Some work has been presented [32] on the effects of lipases on microvesicles, and like ghosts, the microvesicles appear to be much less specific than RBC for each lipase. As a result of these observations, only intact RBC were treated with lipases.

The two lipases used in the work presented here function differently. PLA_2 removes the sn2 acyl-chain and cleaves only 1,2-diacylglycerophospholipids, including PC, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol [27], although in the outer leaflet of the RBC membrane, PC is the preferred substrate. Consistent with this, no significant change in the level of phosphatidylethanolamine could be detected by TLC. The resultant cleavage products, fatty acid and lyso-PC, were removed by the subsequent washes with bovine serum albumin. No lyso-PC could be detected by TLC analysis of extracted lipids; this is consistent with the fact that lysis was < 5% in all our experiments and a report that 2 mol% lyso-PC incorporated in the membrane causes 50% hemolysis of RBC [40]. SMase C, specific for sphingomyelin [27], cleaves the phosphorylcholine group from sphingomyelin, leaving ceramide; more than 90% of the SM in the RBC membrane is in the outer leaflet. According to Verkleij and colleagues [11], the ceramide created by the action of SMase C does not leave the bilayer but accumulates in droplets as detected by electron micrography.

The above observations suggest that the kinetics of cholesterol efflux after treatment with SMase C may be

TABLE I

The effects of phospholipase treatment on cholesterol efflux from RBC

The time-courses were run under the conditions described in Fig. 1. The rate constants were determined by computer modeling, as described in Materials and Methods. Each measurement is the mean (+S.D.) of nine determinations. The $t_{1/2}$ values are not significantly different, as determined by Tukey's test [26].

Treatment	%PL removed (% of control)	Ratio of SM/PC (w/w)	Halftime for FC efflux (h)
Control RBC	0	0.9/1	4.6 ± 0.6
PLA_2-RBC	30–40	1.9/1	4.4 + 0.8
SMase C-RBC	40–50	0.5/1	3.7 ± 0.4

quite complex because cholesterol may be sequestered into a droplet of ceramide. It is difficult to predict what changes in cholesterol-phospholipid interactions would accompany the formation of the ceramide droplets: it is likely that, unlike SM, ceramide molecules partition to the inner leaflet of the RBC membrane. It seems that in the outer leaflet of the RBC membrane, the strength of interaction of cholesterol molecules with host lipid molecules does not change much because the $t_{1/2}$ for exchange of cholesterol to HDL is quite insensitive to the conversion of SM to ceramide. In agreement with the results shown in Fig. 1, Slotte et al. [33] have shown that treatment of fibroblasts with SMase C does not alter cholesterol efflux to HDL. However, there is a redistribution of cholesterol from the plasma membrane of the fibroblasts to internal membranes: this suggests that greater changes in cholesterol-phospholipid packing density occur in the inner leaflet of the plasma membrane.

The lack of change in cholesterol efflux from PLA_2-treated RBC membranes may be a reflection of the high cholesterol content of the membrane. The cholesterol/phospholipid lateral packing density is very high due to the condensation of PL acyl-chains by cholesterol, so that degradation and removal of some of the PL does not change the average molecular packing density significantly. Consequently, the $t_{1/2}$ for cholesterol efflux remains more-or-less constant. Data attained with PL/cholesterol monolayers suggest that when the monolayer is highly condensed due to a high content of cholesterol, the lateral molecular packing density is relatively insensitive to changes in surface pressure [20]. This highly-condensed state of the PL/cholesterol bilayer appears to exist in the RBC membrane. In this situation, variations in acyl-chain composition of the PL are not expected to alter the average cholesterol microenvironment very much; consistent with this concept, the initial rate of efflux of cholesterol from RBC is not significantly altered by manipulating the PC acyl-chain composition (cf. Fig. 1 in Ref. 41). The change in RBC shape on lipase treatment may also help to maintain a constant rate of cholesterol exchange. Thus, various authors [28,34] have suggested that the lipase-treated RBC change shape to accommodate the change in bilayer packing. The loss of PL by treatment with lipases may result in a compensating shrinkage of the membrane, so that the average interactions of cholesterol within the membrane do not change much. Whether or not there are changes in the phospholipid domains [35] of the RBC membrane on lipolysis is not known.

Cytoskeletal proteins

The RBC cytoskeleton is formed mainly by spectrin, actin and band 4.1, while ankyrin anchors the network to the plasma membrane by binding to both band 3 and

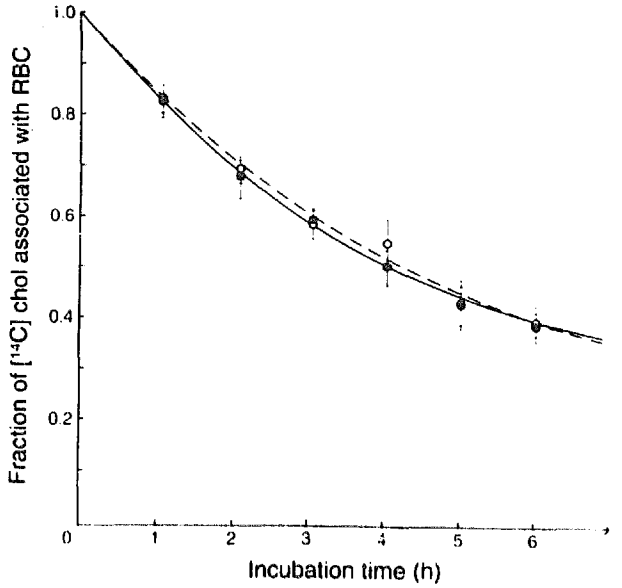

Fig. 2. Time-courses of cholesterol efflux from control and diamide-treated RBC ghosts to human HDL_3. Incubations and conditions were as described for Fig. 1, except that ghost preparations (5.3–7.2 μg cholesterol, 450–1055 cpm [^{14}C]FC/μg FC) were incubated with HDL_3 (final concentration of 0.60 μg FC/μl) in a final volume of 100 μl of buffer 1 + 0.2% BSA. Each point is the mean (± S.D.) of nine determinations. ○------○, control ghosts; ●———●, diamide-treated ghosts.

spectrin. Band 3 (an integral membrane protein) is also associated with glycophorin (another integral membrane protein), which is linked to the spectrin-actin network via band 4.1 [36]. To determine the influence of the cytoskeleton on cholesterol efflux, RBC were treated in two different ways. One approach was to cross-link spectrin (thereby disrupting the cytoskeleton) with diamide [15]; the other approach was to heat-treat the RBC, which disrupts the cytoskeletal attachment points to the membrane. Modifying the RBC cytoskeleton made the RBC very fragile and prone to lysis upon shaking. Due to this lysis, all cholesterol-exchange experiments involving modification of the cytoskeleton were carried out with RBC ghosts.

Since all of the following work was done with RBC ghosts, cholesterol efflux from unmodified RBC ghosts (see Fig. 2 for a time-course) was compared to cholesterol efflux from whole cells. The results, summarized in Table II demonstrate that cholesterol efflux from ghosts (4.2 ± 0.4 h) is comparable to that from whole cells (4.6 ± 0.6 h). Examination of the ghost preparations by SDS-PAGE showed that bands 1 and 2 (spectrin) and 3 (the anion transport protein) are clearly visible, as are some of the minor protein components of the ghosts (Fig. 3). Cross-linking of spectrin by either diamide or heat-treatment created a high molecular weight band of spectrin, as described previously [15].

TABLE II

Cholesterol efflux from different RBC preparations

The time-courses were run under the conditions described in Figs. 1–5. The rate constants were determined by computer modeling, as described in Materials and Methods. The $t_{1/2}$ values are mean values (±S.D.). Each kinetic experiment was run in triplicate. n is the number of cell preparations.

Cell preparation	Halftime for FC efflux (h)	n
Control RBC	4.6±0.6	6
Control ghosts	4.2±0.4	4
Diamide-ghosts	3.6±0.6	5
Heated ghosts	4.0±0.4	4
A23187 microvesicles	4.2±1.9	4

The time-courses of cholesterol efflux from ghosts and diamide-treated ghosts are compared in Fig. 2. The time-courses are similar for the two preparations and the halftimes, which are not significantly different from each other, are listed in Table II. A slight decrease in the $t_{1/2}$ for cholesterol exchange from RBC ghosts after oxidative cross-linking of sulfhydryl groups in membrane proteins by diamide has been reported [37]. The cross-linking of spectrin via intermolecular disulfide coupling [38] decreases the lateral mobility of the band-3 protein. It follows that alterations in the lateral mobility of the latter integral membrane protein have no effect on the ability of cholesterol to leave the RBC membrane. Cross-linking of spectrin by heat treatment also does not alter the $t_{1/2}$ for cholesterol efflux (Table II).

This comparison of cholesterol efflux from RBC, ghosts and diamide- or heat-treated ghosts suggests that disrupting the cytoskeleton (by chemical cross-linking or by heat-treatment) does not significantly influence cholesterol efflux from the membranes. These results imply that the PL-cholesterol molecular packing and interactions in the RBC membrane are not perturbed by the interaction of the cytoskeletal proteins.

Cholesterol efflux from RBC microvesicles

The RBC were also manipulated to obtain membranes in which the proteins and lipids were unrestrained by cytoskeletal attachments. Microvesicles prepared by using either ionophore A23187 [17] or merocyanine 540 [18] tend to retain band 3 (an integral membrane protein) and band 4 (a cytoskeletal protein), but to lose glycophorin (an integral membrane protein) and several of the cytoskeletal proteins (bands 1 and 2, spectrin and band 5, actin), as well as the cytoskeleton itself. The fact that this reorganization occurred with our microvesicles prepared by the above methods is substantiated by the SDS-PAGE data in Fig. 3. The yield of microvesicles obtained with A23187 treatment of RBC was sufficient for a full analysis of the kinetics of cholesterol exchange; the yield with merocyanine treatment was extremely small and only one control cholesterol exchange experiment could by performed with the preparation.

The experimental approach for cholesterol efflux from microvesicles to acceptor HDL_3 was similar to that described above for cholesterol efflux from whole cells or ghosts. A methodology for separation and analysis of the acceptor HDL_3 particles and the microvesicles was developed, because the microvesicles and lipoprotein could not be separated by either centrifugation or filtration. After the specified incubation time, the acceptor HDL_3 (approx. 10 nm diameter) and donor RBC microvesicles (approx. 150 nm diameter) were separated from each other on a small gel filtration column as described in Materials and Methods. Fig. 4 shows a typical profile seen when [^{14}C] cholesterol-labelled microvesicles are separated from [^{3}H]cholesterol-labelled HDL_3. A typical time-course for cholesterol efflux from A23187-microvesicles is shown in Fig. 5. It is apparent from Table II that the $t_{1/2}$ for cholesterol efflux from these microvesicles is the same, within experimental error, as that from control RBC or ghosts. An experiment performed using microvesicles made with merocyanine 540 gave the same $t_{1/2}$ as that reported for A23187-microvesicles in Table II. It follows that detachment of the cytoskeleton and removal of some integral membrane proteins does

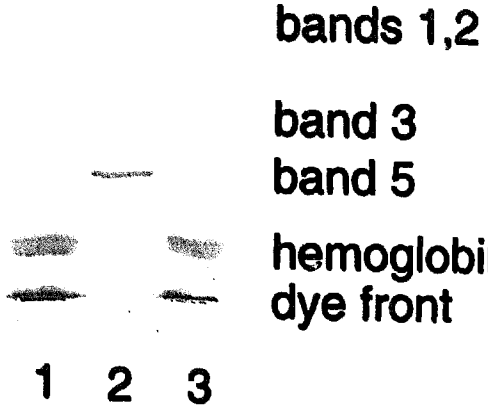

Fig. 3. SDS-PAGE of RBC ghosts and microvesicles. Lane 1 shows a merocyanine-540 microvesicle preparation, lane 2 shows a ghost preparation, while lane 3 shows a A23187 microvesicle preparation. The samples were incubated in running buffer (10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 2.5% SDS, 1% dithiothreitol and 0.01% Bromophenol blue) for 30 min at 37°C and then 4 μg of ghost or 40 μg of microvesicle protein were loaded onto the 4–15% gradient Phastgel. The gel was run at 15°C for 74 V/h with an applied voltage of 250 V and stained with Coomassie blue.

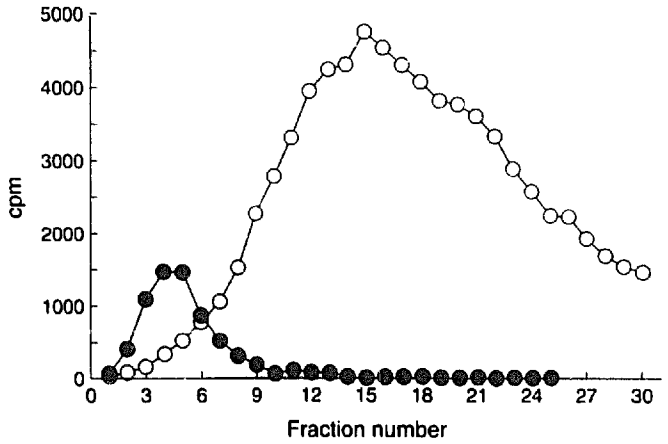

Fig. 4. Separation of A23187 microvesicles from HDL_3 on Sepharose CL-4B. HDL_3 labelled with [^{3}H] cholesterol (O) (65 μg cholesterol, 1800 cpm/μg FC) in a final volume of 100 μl buffer 1 + 0.2% BSA was loaded onto a 2 ml Sepharose-CL-4B column. The column was eluted with buffer 1 + 0.2% BSA and 50 μl fractions were collected and analyzed. A23187 microvesicles labelled with [^{14}C] cholesterol (●) (8 μg cholesterol, 1330 cpm/μg FC) in a final volume of 100 μl buffer 1 + 0.2% BSA were eluted similarly.

not have any consequences for the kinetics of cholesterol efflux from the RBC plasma membrane. Presumably, the cholesterol-phospholipid interactions in the membrane are not significantly affected by the linkage to the cytoskeleton even though the inner leaflet of the membrane is relatively enriched in cholesterol [39]. Consistent with this idea, disruption of the interaction of the membrane skeleton with the bilayer does not affect phospholipid asymmetry in human RBC membranes [16].

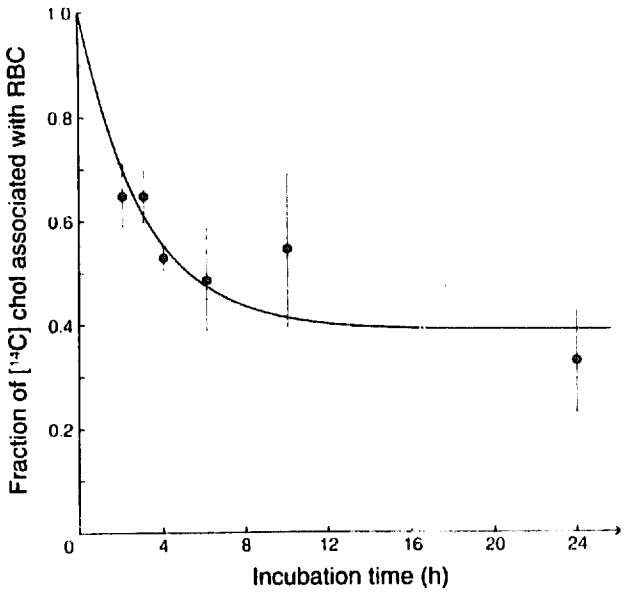

Fig. 5. Time-course of cholesterol efflux from A23187-microvesicles to HDL_3. Microvesicles (6.5 μg cholesterol, 2100 cpm [^{14}C]FC/μg FC) were incubated with HDL_3 (66 μg cholesterol) in a final volume of 100 μl of Buffer 1 + 0.2% BSA. Incubation conditions were as described in Fig. 1. The incubation mixture was separated on a Sepharose CL-4B column as described in Fig. 4. Each point is the mean of three determinations.

Conclusions

It is well-established from model membrane studies that the $t_{1/2}$ for cholesterol exchange is sensitive to the FC/PL interaction energy [19]. In agreement with this, we reported that increasing membrane SM content raises $t_{1/2}$ in simple mixed SM/PC bilayers [4]. However, as judged by comparison of different species of RBC, increasing SM in the RBC membrane was found to have a relatively small effect. The data presented in this paper are consistent in that altering the SM/PC ratio in human RBC by lipolysis does not have a marked effect on $t_{1/2}$. This indicates that although lipolysis inevitably leads to a reorganization of the PL components of the membrane bilayer, the cholesterol packing is apparently not changed much. Consistent with this, the $t_{1/2}$ for cholesterol exchange from different species of RBC is similar although there are variations in the PL compositions [4]. It follows that the RBC membrane is remarkably resilient and can maintain essentially constant FC/PL interactions despite large alterations in phospholipid composition. The high level of cholesterol present in the membrane is probably important for this property because a high FC/PL ratio ensures a condensed lateral packing which is insensitive to moderate alterations in the FC/PL ratio [20]. In addition, changes in membrane shape with variation in lipid composition may play a compensatory role.

The fact that the cytoskeleton can be immobilized or removed with no effect on $t_{1/2}$ indicates that the FC/PL interactions in the outer monolayer of the plasma membrane are not affected by the phospholipid bilayer-protein interactions involved in the attachment of the cytoskeleton. This occurs even though the mobilities of some integral membrane proteins are altered by the manipulation of the cytoskeleton. It seems that for the RBC membrane, which contains a relatively limited complement of integral proteins, the proteins do not modulate the average cholesterol packing in a major way. Presumably, in other cell plasma-membranes which contain a larger variety of proteins, the proteins do modulate the cholesterol packing in the membrane and lead to different cell types having different $t_{1/2}$ values for cholesterol exchange.

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