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The Exchangeability of Human Erythrocyte Membrane Cholesterol

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A new method has been used to determine what fraction of human erythrocyte cholesterol is available for exchange with plasma unesterified cholesterol. Erythrocytes labeled with ^{3}H-cholesterol by this exchange process were incubated with sonicated phosphatidylcholine vesicles, giving rise to a net movement of cholesterol out of the cells. The specific activity of cholesterol taken up by the vesicles depended on the length of time of incubation. Initially the specific activity in the vesicles was greater than that in the cells, but after approximately 10% of cell cholesterol had been removed, the specific activity of subsequently removed cholesterol was equal to that of the remaining erythrocyte cholesterol. We conclude from these data that a) all of the cholesterol in the erythrocyte is exchangeable with plasma, and b) approximately 10% of erythrocyte cholesterol is in a more rapidly exchangeable pool than the remainder.

Key words: **cholesterol exchange, erythrocyte membrane, cholesterol pools**

Since the original studies which showed that unesterified cholesterol readily exchanges between erythrocytes and plasma [1, 2], numerous authors have attempted to determine what proportion of membrane cholesterol participates in this exchange process [3–6]. These measurements all were based on a study of the equilibration of radiolabeled cholesterol between erythrocytes and plasma lipoproteins, all the label being in one or the other component at the outset of the experiment. The results of these studies are divided between those that observed complete exchange of erythrocyte cholesterol [3, 4] and those that found that a portion of erythrocyte cholesterol does not participate in the exchange process [5, 6]. A resolution of these contradictory findings would be of value, as the extent to which erythrocyte cholesterol is available for exchange has important implications with regard to membrane structure.

In a recent paper [7] we showed that the transfer of labeled cholesterol between human erythrocytes and plasma occurred by the same mechanism and involved the same pool of molecules whether the transfer involved no net movement of cholesterol (exchange)

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or whether a net movement of cholesterol out of the cells took place. In spite of the fact that this study did not reveal some of the details of exchange reported here, it provided the basis for a new, extremely simple technique for the determination of the amount of exchangeable erythrocyte cholesterol. In the experiments described in this paper, erythrocytes labeled with ^{3}H-cholesterol by exchange were incubated with sonicated phosphatidylcholine vesicles so as to deplete the cells of cholesterol. Because exchange and depletion are manifestations of the same process, the cholesterol molecules removed from the membrane in this way came from the pool which was labeled by the exchange process and the fraction of total membrane cholesterol which was labeled by exchange could be determined by measuring the specific activity of the cholesterol taken up by the vesicles. This study demonstrates that cholesterol in human erythrocytes is in two distinct kinetic pools, one of which contains approximately 10% of the cell cholesterol and exchanges rapidly with plasma, while the other contains the bulk of cell cholesterol and exchanges with plasma at a slower rate. Furthermore, the results demonstrate that all of human erythrocyte cholesterol is exchangeable with plasma.

METHODS

Blood from healthy human donors was collected in heparin (4,000 USP units/liter blood). Plasma and the "buffy coat" were separated from the cells after 15 min centrifugation at 1,500g. The red cells were washed three times in 10 volumes of the following buffer, which was used in all experiments: 150 mM NaCl, 5 mM KCl, 5.5 mM Na_2HPO_4, 0.8 mM NaH_2PO_4, 0.5 mM $CaCl_2$, 5 mM glucose, pH 7.4. Plasma was preheated for 50 min at 56°C to destroy lecithin:cholesterol acyltransferase activity [8] and then labeled with ^{3}H-cholesterol as described by Murphy [9]. Labeling of erythrocytes with ^{3}H-cholesterol by exchange from plasma was performed as follows. Erythrocytes were incubated at 37°C at a hematocrit of 30% in buffer containing approximately 4% labeled plasma. In some experiments plasma was labeled with ^{3}H-cholesterol "indirectly," as follows. Erythrocytes labeled by exchange from autologous ^{3}H-cholesterol plasma were washed extensively in autologous unlabeled plasma and then incubated with a fresh aliquot of the same plasma for 16 h. This plasma then was used to label erythrocytes to be used in the experiment. Low density lipoproteins (LDL) were isolated from the plasma of fasting human donors using established procedures [10]. LDL was labeled by incubation with ^{3}H-cholesterol-labeled erythrocytes for 16–18 h.

Egg phosphatidylcholine (grade I) was purchased from Lipid Products (Redhill, Surrey, UK) and gave a single spot after thin-layer chromatography on silica gel. Vesicles were prepared by lyophilization of the lipid from benzene, dispersion in buffer, and sonication for 20 min with a Branson sonifier under N_2 in a water-jacketed cell at 4°C. The sonicate was centrifuged at 15,000g for 20 min to remove titanium particles shed from the probe.

"Inside out" membrane vesicles (IOVs) were prepared and assayed for sidedness as described by Steck [11]. Sidedness and sealing of IOV preparations were assessed before and after incubation with phosphatidylcholine vesicles using acetylcholinesterase as a membrane marker. Purity was between 80 and 90%. Incubations of erythrocytes or IOVs labeled with ^{3}H-cholesterol with sonicated vesicles were carried out at a hematocrit of approximately 20% in a shaking water bath at 37°C. Streptomycin sulfate was added as an antibacterial agent. Approximately 15 μM vesicle phospholipid was added to 5 μM membrane phospholipid. After 1–3 h the vesicles were removed for the assay of the specific

activity of the cholesterol they had taken up and replaced with a fresh aliquot of the same vesicle preparation. By incorporation of ^{14}C-labeled phosphatidylcholine into the vesicles during their preparation, it was shown that no significant loss of vesicles from the supernatant occurred under the conditions of these experiments. Incubations were terminated by centrifugation for 10 min at 1,500g or for 15 min at 17,000g to pellet the erythrocytes and IOVs respectively. The supernatant containing the vesicles then was centrifuged for 20 min at 27,000g to remove any membrane fragments resulting from the small amount of hemolysis which occurred in some experiments. This supernatant then was concentrated under N_2 in a magnetically stirred cell using an Amicon Diaflo ultrafilter and the lipids were extracted [12]. Erythrocyte lipid extraction and cholesterol assays were performed as described [7]. Due to the large amount of phospholipid present in the supernatant lipid extracts, it was found necessary to carry out a saponification step followed by hexane extraction prior to cholesterol assay of these samples. Radioactive PC, the generous gift of Dr Trevor Redgrave, was synthesized from enzymatically prepared phosphatidic acid and choline chloride (methyl-^{14}C) [13]. ^{3}H-cholesterol and ^{14}C-choline chloride were purchased from New England Nuclear, Boston, Massachusetts.

RESULTS

When erythrocytes labeled with ^{3}H-cholesterol by exchange from plasma were incubated with egg lecithin vesicles, cholesterol was taken up by the vesicles, in consonance with the findings of others [14]. The specific activity of the cholesterol removed from the erythrocytes was found to depend on the time of incubation, that is, on the extent of cholesterol depletion. To obtain precise data on the specific activity changes, vesicles were incubated with erythrocytes until they had taken up sufficient cholesterol to permit the assay of specific activity and then were replaced with fresh vesicles to remove more erythrocyte cholesterol. It was found that the cholesterol initially removed from the cells had specific activity higher than that of the cholesterol in the erythrocytes. As more cholesterol was removed from the cells, the specific activity of this cholesterol decreased until it was equal to that of the remaining erythrocyte cholesterol (see experiment 1, Table I and Fig 1a). The equality of specific activity of removed cholesterol and remaining cholesterol was reached after the removal of approximately 10% erythrocyte cholesterol.

Several control experiments were carried out to rule out the possibility that the high-specific-activity cholesterol removed initially from the erythrocytes represented a highly labeled plasma component that had remained stuck to the cell surface in spite of the washes carried out after the incubation to exchange ^{3}H-cholesterol into the cells from plasma. Cells that had been labeled by incubation with ^{3}H-cholesterol labeled plasma were washed thoroughly with unlabeled plasma, and these cells – putatively freed of any adsorbed labeled plasma – were used to label a further aliquot of plasma. In turn this plasma was used to label erythrocytes, which then were incubated with vesicles to remove cholesterol. The high specific activity of initially removed cholesterol still was observed (experiment 2, Table I and Fig 1a). A similar method was used to label purified human plasma low-density lipoprotein, which then was used to label erythrocytes. This method of labeling erythrocytes prior to cholesterol depletion also gave the same result (experiment 3, Table I and Fig 1a).

We conclude that the high-specific-activity cholesterol initially removed from erythrocytes in the depletion experiments is a manifestation of a true membrane phenomenon.

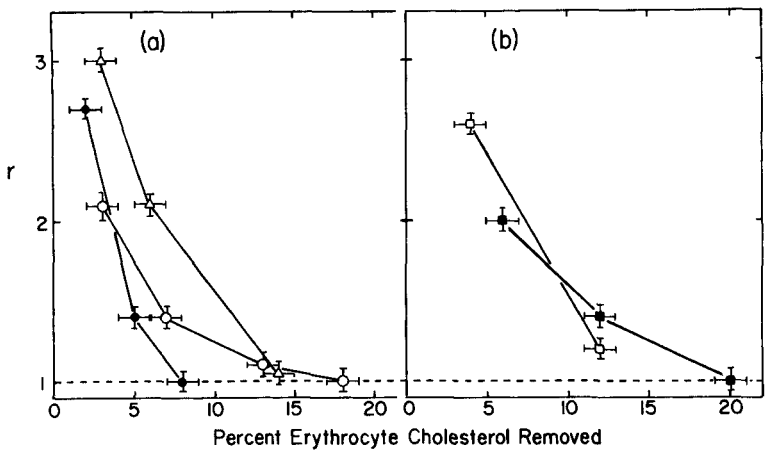

Fig 1. Results of cholesterol depletion experiments using a) intact erythrocytes (RBC) labeled directly from plasma (○); indirectly from plasma (●); from LDL (△); b) IOVs prepared from labeled RBC (■) or labeled after preparation (□). r is the cholesterol specific activity in vesicles at the end of an incubation, expressed relatively to the final specific activity in RBCs after an experiment consisting of 2–4 sequential incubations of vesicles with membranes. For each incubation the value of r is plotted versus the percentage of membrane cholesterol which had been removed by the end of that incubation.

TABLE I. Specific Activity of Cholesterol Removed From Erythrocytes (RBC) and Inside-Out Membrane Vesicles (IOV) by Incubation With Vesicles*

Experiment no.	Membrane system	Labeling method	Incubation no.	% RBC cholesterol removed	Specific activity (10^5 dpm/mg cholesterol) Vesicles	Initial RBC	Final RBC	r
1	Intact RBC	Direct plasma	1	3	16.9	9.1	8.2	2.1
			2	7	11.6			1.4
			3	13	9.0			1.1
			4	18	8.1			1.0
2	Intact RBC	"Indirect" plasma	1	2	12.4	5.1	4.6	2.7
			2	5	6.3			1.4
			3	8	4.6			1.0
3	Intact RBC	LDL	1	3	5.7	2.1	1.9	3.0
			2	6	4.1			2.1
			3	14	2.2			1.1
4	IOV	Labeled after preparation	1	4	56.2	25.4	21.7	2.6
			2	12	25.2			1.2
5	IOV	Prepared from labeled RBC	1	6	15.3	8.6	7.5	2.0
			2	12	10.3			1.4
			3	20	7.6			1.0

*Vesicles were incubated with membranes for 1–3 h, removed, and replaced with fresh vesicles. Column 5 shows the amount of cholesterol removed from RBC by the end of a given incubation with vesicles. The specific activity of the cholesterol taken up by the vesicles during each incubation is given in column 6 and the ratio (r) of this specific activity to the final RBC specific activity is given in column 9.

The experimental findings can be described by the following model. Erythrocyte cholesterol exists in two kinetically distinct pools, one of which contains approximately 10% of the total cholesterol and exchanges more rapidly with plasma cholesterol than does the other pool, which constitutes approximately 90% of erythrocyte cholesterol. During the initial incubation, in which ^{3}H-cholesterol was exchanged into the cells for a period of 16–19 h, the small, more rapidly exchangeable pool was closer to equilibrium with plasma cholesterol than was the larger pool, and the former therefore had reached higher specific activity at the end of the incubation. The cholesterol initially removed from the cells by incubation with vesicles came from the more rapidly exchangeable pool and therefore had high specific activity. After the pool was emptied out, cholesterol removed subsequently had lower specific activity, reflecting the presence of the larger, more slowly exchangeable pool.

We investigated the effect of the duration of the incubation to label the erythrocytes on the specific activity of cholesterol in the two pools. It was found that doubling the labeling period from 10 to 22 h did not alter the specific activity of the cholesterol initially taken up by the vesicles, that is, the cholesterol in the smaller pool (see experiment 6, Table II). However, the specific activity of cholesterol in the larger pool increased somewhat. These results are consistent with the model, since the small pool already had equilibrated with plasma during the 10-h incubation, whereas the specific activity of the large pool continued to increase between 10 and 20 h.

The following experiment was carried out to determine whether any exchange occurred between the two cholesterol pools. Erythrocytes were labeled by incubation with ^{3}H-cholesterol-labeled plasma for 6 h. An aliquot of these cells was incubated immediately with lecithin vesicles to sample the specific activity of the cholesterol in the small pool. Another aliquot of the same labeled cells was kept in buffer at 37°C for 18 h. After this delay the cells were incubated with lecithin vesicles to sample the specific activity of the cholesterol in the small pool. The results of this experiment are shown in Table II (experiment 7). It can be seen that the specific activity of the cholesterol in the

TABLE II. Time Dependence Studies of Specific Activity of Cholesterol Removed From Erythrocytes by Incubation With Sonicated Vesicles*

Experiment no.	Time of labeling incubation (h)	Incubation no.	% RBC cholesterol removed	Vesicles	Specific activity (10^5 dpm/mg cholesterol) Initial RBC	Final RBC	r
6	10	1	3	28.6	16.3	14.6	2.0
		2	8	17.0			1.2
		3	14	14.3			1.0
	22	1	5	29.2	24.1	20.9	1.4
		2	10	20.3			1.0
7	6 No delay	1	4	41.3	20.6	ND	
	18-h delay	1	6	35.2			

*See text and caption of Table I for explanation. In experiment 6 the durations of the incubations with sonicated vesicles were the same in both samples.
ND: Not determined.

small pool had decreased by approximately 15% as a result of the 18 h delay period. Even in the absence of any mechanism within the erythrocyte for exchange between the two pools, one would expect some decrease in the specific activity of the small pool due to exchange between erythrocytes. It follows that cholesterol exchange between the two pools occurs extremely slowly, if at all.

To determine whether the small cholesterol pool is accessible from both sides of the membrane, cholesterol depletion experiments were carried out with the inside-out membrane vesicle preparation described by Steck [11]. It was found that whether the membrane vesicles were prepared from ^{3}H-cholesterol labeled erythrocytes or labeled after their preparation from unlabeled erythrocytes, the specific activity of the cholesterol removed from the membrane depended on the amount of cholesterol removed in a manner qualitatively similar to that observed in intact erythrocytes (see experiments 4 and 5, Table I and Fig 1b).

It is possible that rather than reflecting two cholesterol pools in each erythrocyte, these experiments indicate two types of cells – 10% of the cells having very fast exchange characteristics compared to the rest. Differences in cholesterol exchange due to the age distribution in the erythrocyte population were investigated as follows. Erythrocytes were incubated in ^{3}H-cholesterol-labeled plasma for a period of 1 h – a time chosen so as to label predominantly the rapidly exchangeable pool. The cells then were centrifuged at 37,000g for 1 h in a tube 7 × 700 mm – a procedure which leads to the separation of erythrocytes according to age, the youngest cells having the lowest density [15]. Erythrocytes in the top 10% and the bottom 10% fractions of the tube were extracted and the specific activity of their cholesterol was determined. No difference between the fractions was observed.

DISCUSSION

Previously reported measurements of erythrocyte exchangeable cholesterol were based on experiments in which erythrocytes and plasma lipoproteins were incubated together until equilibration of labeled cholesterol, initially present in one or the other component, had occurred [3–6]. Such experiments necessitate lengthy incubations (up to 24 h) and suffer from the further disadvantage that knowledge is required of how much of the unesterified cholesterol in plasma is exchangeable. In contrast, the experiments described in this paper involve only relatively short incubations of erythrocytes and provide a precise and sensitive measure of the size of the exchangeable cholesterol pool.

In principle, a careful study of the kinetics of ^{3}H-cholesterol exchange between erythrocytes and lipoproteins would reveal the presence of the rapidly exchangeable pool we report here. However, the fact that this pool constitutes only about 10% of total cell cholesterol would make it difficult to observe in such a study, and it is therefore not surprising that it has not been reported hitherto.

There are two possible explanations for our findings. First, it is possible that the cell population is not homogeneous with respect to the characteristics of cholesterol exchange. We have shown that the rapidly exchangeable pool cannot be accounted for in terms of an inhomogeneity in cholesterol exchange characteristics due to cell age. Further study is needed to determine whether some other cell population variable could be involved.

The alternative possibility is that erythrocyte membrane cholesterol exists in two pools within the cell membrane. The finding that the small pool is still present in IOVs suggests that it is associated with the membrane bilayer. It seems unlikely that the small pool is cholesterol bound to intrinsic membrane protein, although this possibility cannot

be ruled out at present. It has been reported recently that band 3 protein from human erythrocytes interacts strongly with cholesterol [16]. However, all evidence to date suggests that lipid associated with intrinsic membrane proteins, although in a different state from bulk membrane lipid, is readily exchangeable with other membrane lipid [17, 18]. Further study is required to determine if the more rapidly exchangeable molecules differ from the rest of the cell cholesterol in their interaction with some other membrane constituent or if they form an isolated cholesterol pool within the membrane.

Our studies of cholesterol depletion of ^{3}H-cholesterol-labeled erythrocytes have shown that after the removal of the small amount of high-specific-activity cholesterol, subsequently removed cholesterol has the same specific activity as that of the erythrocytes. This demonstrates that all of the erythrocyte cholesterol must have been labeled and therefore that it all is exchangeable with plasma. Previous studies [19, 20] have shown that transbilayer movement of cholesterol occurs in the human erythrocyte membrane with a half-time of less than 50 min at 37°C. Since cholesterol exchange occurs at the outer half of the membrane bilayer and it is extremely unlikely that all membrane cholesterol is located in this half of the membrane, the finding of the present paper indicates that cholesterol in both halves of the membrane is exchangeable with plasma. This provides further demonstration, based on an entirely different experimental approach, that cholesterol can move between the two halves of the bilayer in the erythrocyte membrane.

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