ORIGINAL

Alterations in erythrocyte membrane lipid and its fragility in a patient with familial lecithin : cholesterol acyltrasferase (LCAT) deficiency

Takeo Suda[1], Akira Akamatsu[2], Yutaka Nakaya[1], Yasunobu Masuda[3] and Junzo Desaki[4]

[1]*Department of Nutrition, The University of Tokushima School of Medicine, Tokushima, Japan ;* [2]*Department of Internal Medicine, The Matsuyama Prefecture Hospital, Ehime, Japan ;* [3]*R & D Division of Q.P. Co., Fuchu-Shi, Tokyo, Japan ; and* [4]*Department of Anatomy, The Ehime University School of Medicine, Ehime, Japan*

Abstract : Lecithin : cholesterol acyltrasferase (LCAT) plays a key role in the cholesterol metabolism-mediated esterification of free cholesterol into the cholesterol ester in normal plasma. Familial LCAT deficiency is frequently associated with anemia. Using biochemical and physiological techniques, the erythrocytes of this patient were investigated to gain an insight into the relationship between the abnormalities of lipid metabolism and erythrocyte membrane fragility. Abnormal erythrocytes, so-called Target cells and/or Knizocytes, were observed at 20% in our patient's erythrocytes. Moreover, the mean corpuscular volume of the patient's cells was 7% greater than that of a normal individual. In the membrane lipids of the patient's erythrocytes, cholesterol and phosphatidylcholine increased, and phosphatidylethanolamine decreased. The electron spin resonance technique with a fatty acid spin probe showed that the membrane fluidity was more elevated than that of normal cells in spite of the increase in cholesterol content and the cholesterol/ phospholipid ratio of the membrane of patient's erythrocytes. The patient's abnormally shaped erythrocytes were less deformed than those of the normal individual under high shear stress. The partial depletion of membrane cholesterol from the patient's erythrocytes was demonstrated by incubation with normal plasma with LCAT activity. The increment of transformed erythrocytes during the incubation could be prevented by cholesterol depletion from the patient's erythrocyte membrane. These findings indicate that normochromic anemia of the patient might be caused by erythrocyte fragility resulting from decreased deformity and/or abnormal shape of the cells due to abnormal lipid composition in the membrane.
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Keywords **:** *familial lecithin : cholesterol acyltrasferase deficiency, cholesterol, osmotic fragility, membrane fluidity, erythrocyte deformability.*

INTRODUCTION

Since the original description by Norum and Gjone in 1967 (1), only 14 families with hereditary lecithin : cholesterol acyltrasferase (LCAT) deficiency have been reported in Japan. This rare disease is

Abbreviations : LCAT ; lecithin : cholesterol acyltrasferase, the CE/TC ratio ; the cholesterol ester/total cholesterol ratio, the C/P ratio ; the free cholesterol/total phospholipids ratio, HDL ; high density lipoprotein, ESR ; electron spin resonance, I(5.10) : a stearic acid analogue of spin probe

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Address correspondence and reprint requests to Yutaka Nakaya, M.D., Department of Nutrition, The University of Tokushima School of Medicine, Kuramoto-cho, Tokushima 770-8503, Japan and Fax : +81-88-633-7113.

manifested by moderate anemia, proteinuria and corneal opacity (2-5). The proportion of cholesterol esters in the total plasma cholesterol is invariably depressed, although total cholesterol concentration may either be depressed or elevated. It is also known that cholesterol accumulates in a limited number of organs in patients with genetic or secondary LCAT deficiencies (6, 7), and erythrocytes are one of the primary organs where such cholesterol accumulation takes place.

LCAT specifically converts free cholesterol and phosphatidylcholine into cholesterol esters and lysophosphatidylcholine, respectively (8). Furthermore, this conversion is an irreversible reaction in plasma as follows : membrane free cholesterol ←- -→ plasma free cholesterol - -→ plasma cholesterol ester. Hence its deficiency impairs the transport of cholesterol from extrahepatic tissues to the liver via plasma cholesterol esters. The resulting changes of membrane lipids and/or erythrocyte shape are then presumed to be responsible for the instability of the patient's erythrocytes with mechanical strain in circulation. In this report, we examined a typical case of this disease emphasizing the role of membrane lipids in the physical properties of the erythrocytes. Contrary to the above presumption, the patient's erythrocytes exhibited decreased osmotic fragility, and increased membrane fluidity and visco-elasticity despite the fact that the majority of the cells were transformed, presumably owing to the abnormal intramembrane distribution of lipid components as a result of the lipid equilibrium.

Case presentation :

A 31-year-old woman exhibited the typical symptoms of LCAT deficiency, such as anemia, proteinuria and corneal opacity, but showed no liver or renal dysfunctions. She had been well until her visit and her family history is summarized in Fig. 1. Hematocrit was 32.7 percent ; red-cell count 3.29×10^6 per cubic millimeter, white-cell count 8,100 per cubic millimeter, and platelet count 200,000 per cubic millimeter. The blood levels of total cholesterol was 96 mg/dL, triglyceride 204 mg/dL, HDL-choesterol 13 mg/dL, total bilirubin 1.7 mg/dL, total protein 7.7 g/dL, aspartate aminotransferase 20 IU, alanine aminotransferase 26 IU, alkaline phosphatase 191 IU and creatine kinase 111 IU. LCAT activity was 30% of the control. Informed consent was obtained from the patient and her family.

Fresh blood was drawn by venipuncture into a heparinized tube, and erythrocytes were washed twice with an isotonic phosphate buffer containing 42.6 mM Na_2HPO_4, 7.2 mM $NaHPO_4$. 5.1 mM KCl, 90.3 mM NaCl and 5.6 mM D-glucose, pH 7.4. The cells were kept at 4 °C.

Lipid and protein analysis

Lipids were extracted from the plasma and erythrocyte membrane with 10 volumes of $CHCl_3$-methanol (2/1 by volume). Plasma cholesterol was measured by the colorimetric method, and that in the erythrocyte membrane was determined by gas chromatography as described previously (9, 10). Phospholipid compositions were analyzed by two dimensional thin layer chromatography with Silica-Gel H (Merk Co.

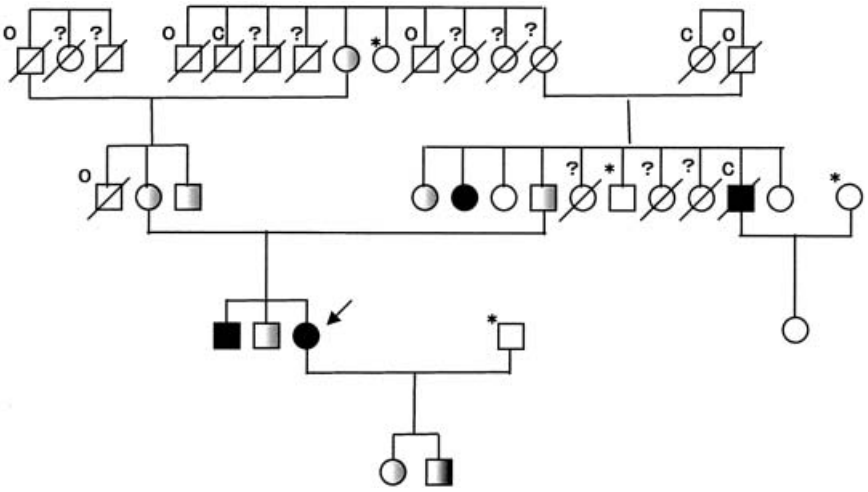

Fig. 1. Pedigree of familial LCAT deficiency. Arrow indicates proband. Circles denote female subjects ; squares denote male subjects. Arrows indicate proband, and diagonal lines indicate deceased subjects. At the top left of the symbols, the cause of death is indicated, i.e., C indicates cardiovascular disease, O other diseases, ? unknown cause. In the symbols, black represents subjects with typical LCAT deficiency (LCAT activity>39% of normal), black and white represents partial deficiency (LCAT activity 40-60% of normal), and white indicates unaffected subjects. *indicates patients who were not studied.

Whitehouse Station, NJ), which was developed with $CHCl_3$-methanol-glacial acetic acid-water (25/15/4/2) and $CHCl_3$-methanol-28% ammonia-water (120/15/4/2) as the first and second developing solvents, respectively. The fatty acids of the membrane phospholipids were analyzed by gas chromatography after being esterified with 10% HCl-methanol (Tokyo Kasei Co., Tokyo, Japan) Lecithin : cholesterol acyltransferase activity was determined using a commercial test kit (Daiichi Chemicals Co, Ltd Tokyo, Japan) (11).

Scanning electron microscopy

Erythrocytes of the patient and a normal individual (33-year-old man) were fixed with 1% glutaraldehyde and 1% OsO_4, and successively dehydrated with ethanol. Then, the specimen was coated with Pt using an Ion Coater (Model IB-5, Eiko Engineering., Tokyo, Japan) and observed with a scanning electron microscope (Model S-500, Hitachi Co. Tokyo, Japan).

Effect of membrane cholesterol depletion

In order to investigate the relationship between membrane cholesterol and the erythrocyte shapes of the patient, the cells were incubated with normal plasma with LCAT activity (12). The erythrocytes were diluted to 10% Ht, with plasma containing Penicillin G (1000 units/ml) as a sterile drug, and incubated for six hours at 37°C. After incubation, the test tubes were centrifuged to remove the plasma, and the lipids and hemoglobin were measured. Over six hours, no hemolysis could be detected by the cyanmethemoglobin method. Cells were washed twice with isotonic buffer to analyze the membrane lipids and to observe the erythrocyte shapes.

Membrane fluidity measurement

A stearic acid analogue of spin probe, 2-(10-carboxydecyl)-2-hexyl-4, 4'-dimethyl-3-oxazolidinyloxyl, was purchased from Syva Co. (Palo Alto. CA). The spin probe was incorporated into intact erythrocyte membranes by labeled albumin as described previously (8). The labeled cells were packed in a capillary tube, and electron spin resonance (ESR) spectra were recorded by a Varian E-3 spectrometer at various temperatures.

Measurement of erythrocyte deformability and osmotic fragility

The cells were suspended in isotonic buffer with 20% Dextran T-40 (Pharmacia Chemical Co., Peapack, NJ) and erythrocyte deformability was measured using a rheoscope at 24°C. The ratio of long to short axis lengths was measured as the deformation index. To measure osmotic fragility, the continuous dilution method by Maeda *et al*. (13) was employed, and salt concentrations giving 50% hemolysis and a slope of 25% and 75% hemolysis were subsequently measured.

RESULTS

Hematological examination :

The hematological results are shown in Table 1,

Table 1. Hematological examinations of whole blood

	Normal Individual	LCAT deficiency
Red blood cells ($\times 10^6$/mm^3)	5.37 (4.98)	3.82 (3.80)
Hematocrit (%)	47.6 (45.5)	35.7 (38.0)
Hemoglobin (g/dL)	16.7	12.2
Mean corpuscular volume	89 (91.5)	94 (100)
Mean corpuscular hemoglobin (pg)	31.9	31.8
Mean corpuscular hemoglobin concentration (%)	34.9	33.8
Reticulocytes (%)	1.6	4.3
Platelet ($\times 10^4$/mm^3)	16.4	18.2
White blood cells ($\times 10^3$/mm^3)	7.8	7.3
Neutorphils	60.5	73.6
Lymphocytes	28.5	19.5
Monocytes	4.5	3.5
Eosinophils	5.0	3.0
Basophils	1.0	0.5
Atypical lymphocytes	0.5	not detectable

Measurement by an Coulter Counter (Type A). Values by standard manual methods were given in parenthesises.

in which the changes were mainly in erythrocytes. The mean corpuscular volume increased by 7%. The patient's erythrocytes showed the typical figures of normochromic anemia associated with increased reticulocytes (4.3%). Moreover, the erythrocytes were heterogeneous in shape and about one-fifth of the cells were either so-called Target cells or Knizocytes (Fig. 2).

Comparison of plasma and erythrocyte membrane lipids :

The lipid composition of the patient's plasma is listed in Table 2. Plasma CE concentration was markedly decreased in association with the decrease of TC concentration. In our case, the CE/TC (cholesterol ester/total cholesterol) ratio by weight was decreased to 0.31, whereas the normal value was 0.73. Inversely, the cholesterol content in the erythrocyte membrane of the patient was increased by 140% compared with that of the normal individual (Table 3). Although the changes in phosphatidylchone and phosphatidylethanolamine concentrations in the plasma were small, the difference in plasma total phospholipid concentrations between the patient and the normal subject was maintained (Table 2). The erythrocyte membrane of the patient had increased phosphatidylchone, and decreased phosphatidylethanolamine (Table 3) in a type of equilibration of these phospholipids be-

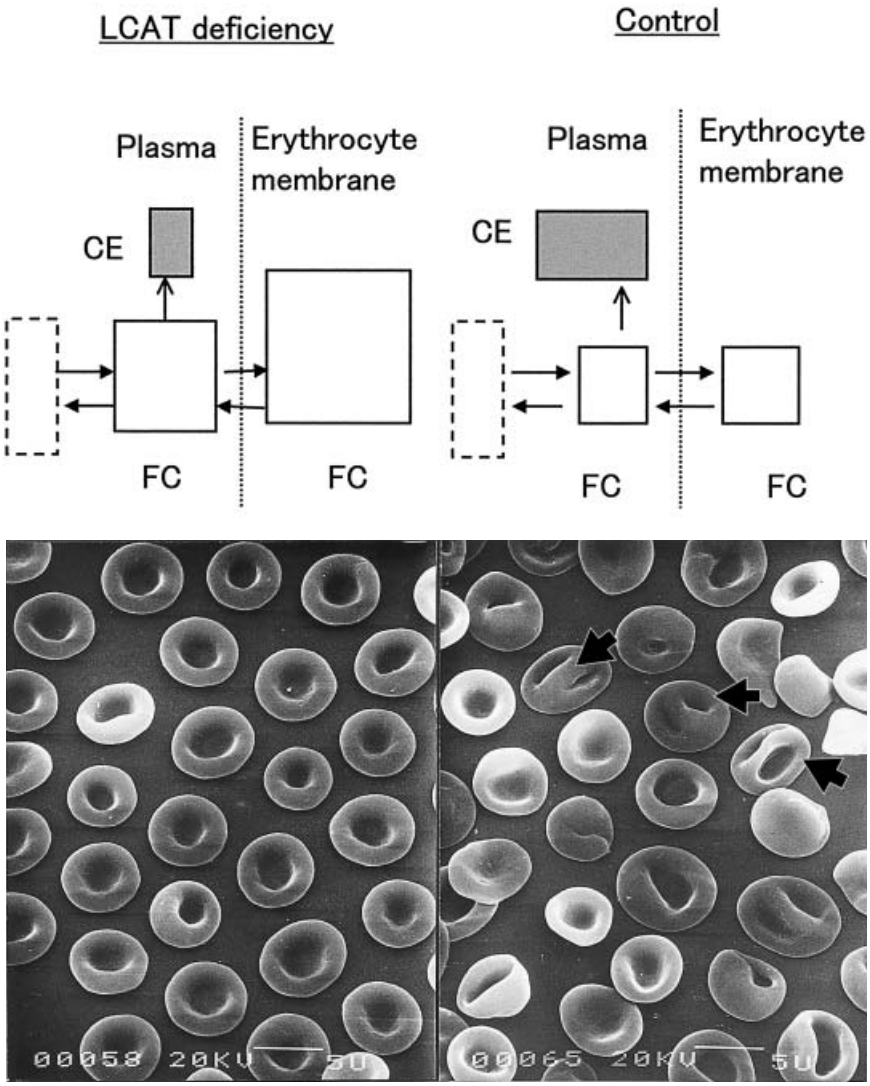

Fig. 2. Schematic illustration of lipid equilibrium (upper panel) and the abnormal shapes of the lipid accumulated erythrocytes in the patient's membrane (lower panel).

Upper panel ; Cholesterol level of the erythrocyte membrane was estimated by the number of erythrocytes in the blood and cholesterol per cell in the membrane. Dotted square indicates other compartments (such as cell membrane or lipoproteins). In a patient with LCAT, cholesterol ester in the plasma was decreased but cholesterol of the erythrocyte membrane increased.

Lower panel ; Left, control subject. Right, LCAT deficiency subject (#1). Abnormal erythrocytes (Target cells) are indicated by arrow heads. Note that the concavity of the cells was lost in Target cells. The white bar in the electron scanning microscope photographs indicated the scale of 5 μm (Magnification × 2800).

Table 2. Plasma lipid of the pateints and naormal individual

	Normal individual	LCAT deficiency
Total cholesterol (TC ; mg/dL)	157	67
Free cholesterol (mg/dL)	43	46
Cholesterol ester (CE ; mg/dL)	114	21
CE/TC	0.73	0.31
Phospholipids (mg/dL)	168	171
Phospholipids composition (%)		
Lysophosphatidylcholine	7.9±2.2	not detectable
Posphatidyliositol+Phsophatidylserine	3.2±0.7	not detectable
Sphingomyelin	17.9±1.1	16.3
Phosphatidylethanolamine	6.1±0.6	1.1
Phosphatidylcholine	64.9±0.2	82.6

Table 3. Erythrocytes membrane lipids of the patient and normal individual

	Normal individual	LCAT deficiency
Cholesterol (C ; $\times 10^{-16}$mol/cell)	3.01±0.08	4.24±0.21
Phospholipids (P : $\times 10^{-16}$mol/cell)	3.66±0.12	4.28±0.11
C/P ratio	0.82	0
Phospholipids composition (mol ; %)		
Lysophosphatidylcholine	0.8±0.7 (0.03)	1.5±0.3 (0.06)
Posphatidyliositol	2.8±0.6 (0.10)	2.5±0.9 (0.11)
Phsophatidylserine	11.8±0.6 (0.43)	9.3±0.5 (0.40)
Sphingomyelin	30.3±2.1 (1.11)	26.0±1.8 (1.11)
Phosphatidylethanolamine	21.5±2.6 (0.79)	9.1±1.7 (0.39)
Phosphatidylcholine	32.7±0.2 (1.21)	51.6±1.6 (2.21)

Values in the parenthesises were calculated from 3.66 and 4.28×10^{-16}moles/cell, respectively.

tween the plasma and erythrocyte membrane in the patient's blood. To obtain more quantitative figures, the phospholipid content in the membranes was calculated on the basis of moles of phospholipids per cell. The phosphatidylchone content per cell in the patient's membrane was 158%, whereas the phosphatidylethanolamine content was 42% of that found in the membrane of the normal individual. However, sphingomyelin and phosphatidylserine in the patient's membrane did not differ. Overall, the ratio of free cholesterol to total phospholipids (C/P) was slightly increased in the erythrocyte membrane of the patient to maintain the lipid partition equilibrium.

Relationship of membrane cholesterol and cell shape :

When normal plasma containing LCAT was added to the patient's erythrocytes, the cholesterol content in the patient membrane decreased, 3.74×10^{-16} moles per cell associated with increase of cholesterol ester concentration in the normal plasma (Table 4). Namely, part of the membrane cholesterol of the patient's erythrocytes moved to the plasma cholesterol ester via plasma free cholesterol by LCAT reaction. When authentic plasma or normal plasma with LCAT inhibitor (iodoacetoamide ; 1 mM) was added to the patient's cells, no cholesterol movement from the membrane occurred. While transformed cells were 28.7% from the influence of long-term erythrocyte incubation of the normal subject, the transformed cells increased from 19.9% to 49.5% in the patient with authentic plasma. This marked increment of abnormal transformation in the patient's cells was held at 27.5% by incubation with normal plasma instead of that of the patient, which was similar to that of the normal subject. This suggested that the prevention of abnormal transformation of the patient's cells might attribute to partial depletion of the membrane cholesterol.

Physical properties of the erythrocytes :

The patient's erythrocyte exhibited marked osmotic resistance. NaCl concentrations at 50% hemolysis were 0.309±0.00 l% and 0.362±0.004% of NaCl equiva-

Table 4. Relationship of membrane cholesterol and erythrocyte shapes

	Erythrocyte Lipids			Transformed Erythrocytes[c]	Plasma Lipids	
	FC[a]	PLs[a]	C/P[b]		FC/TC[d]	CE/TC[d]
Normal subject's erythrocytes						
1) before incubation	3.15	3.80	0.83	3.4±0.4	33.5	66.5
2) incubation with normal plasma	2.79	3.87	0.72	28.7±6.1	29.3	70.7
3) incubation with patient plasma	3.21	3.77	0.85	25.6±8.7	67.6	32.4
4) incubation with normal plasma and LCAT inhibitor	3.22	3.76	0.86	echinocyte	33.7	66.3
LCAT deficiency's erythrocytes						
1) before incubation	4.15	4.33	0.96	19.5±10.0	70.6	29.4
2) incubation with patient plasma	4.22	4.33	0.97	49.5±11.2	69.1	30.9
3) incubation with normal plasma	3.74	4.32	0.91	27.5±9.1	28.9	71.1
4) incubation with patient plasma and LCAT inhibitor	4.23	4.38	0.97	echinocyte	70.2	29.8

a) All values were expressed as $\times 10^{-16}$ moles/cell.
b) All values were expressed as molar ratio.
c) All values were expressed as % and mean±S.D..
d) All values were expressed as weight %.

lent in the patient's and normal cells, respectively. However, the other parameters of osmotic resistance (H25-75) were increased 1.75 fold in spite of the high C/P ratio of the membrane.

The erythrocyte membrane fluidity of the patient was assessed by ESR analysis using a lipid analogue. The erythrocyte membrane gave a small but narrow overall splitting (2T//) between the outer extremes of the ESR spectrum (Fig. 3). This difference was approximately two gausses at 17°C (Fig. 3). When the temperature dependence of the splitting was examined, this difference was evident over the range 2°C and to 39°C. Moreover, the discontinuity seen with a normal membrane at 22°C singularly disappeared in the patient's membrane. The results suggested that the erythrocyte membrane with LCAT deficiency was more fluid in spite of its high cholesterol content.

In order to examine the functional properties of the patient's erythrocytes, the deformability of the cells was measured (Fig. 4). The decreased deformability of the patient's cells, which gave smaller values for deformation indices than normal cells, might reflect increased reticulocytes (4.3%) in the patient's circulation (Table 1).

DISCUSSION

Increased phosphatidylchone and cholesterol, and decreased phosphatidylethanolamine in the patient's erythrocyte membrane (Table 3) are consistent with earlier reports of LCAT deficiency. In our case, the free cholesterol concentration in the plasma was maintained to the normal concentration though the cholesterol ester proportion was low due to LCAT deficiency (Table 2). Inversely, the membrane cholesterol (140%) and phospholipids (127%) in the patient's cells were increased in spite of a decrease of phosphatidylethanolamine (42%) as shown in Table 3. In order to investigate the relation between plasma and the membrane lipid of the patient, we estimated the total amount of free cholesterol (6 l mg/dl) in plasma and the erythrocytes in blood in circulation. As a result, this value was unchanged between the patient's and normal blood. From the above calculation, it was suggested that an equilibrium of cholesterol between the plasma and membrane might exist and decline to the patient's membrane accompanied with increased phospholipids and changes of the phospholipid compositions of the membrane. Thus, membrane cholesterol accumulated accompanied with changes of phosphatidylcholine and phosphatidylethanolamine levels in the membrane.

Owing to abnormality in the membrane lipids, the patient's erythrocytes also exhibited increased mean corpuscular volume, abnormal erythrocyte shapes, and anemia associated with increased reticulocytes. Concerning osmotic resistance *in vitro*, our previous report (9, 13) showed that the C/P ratio of the mem-

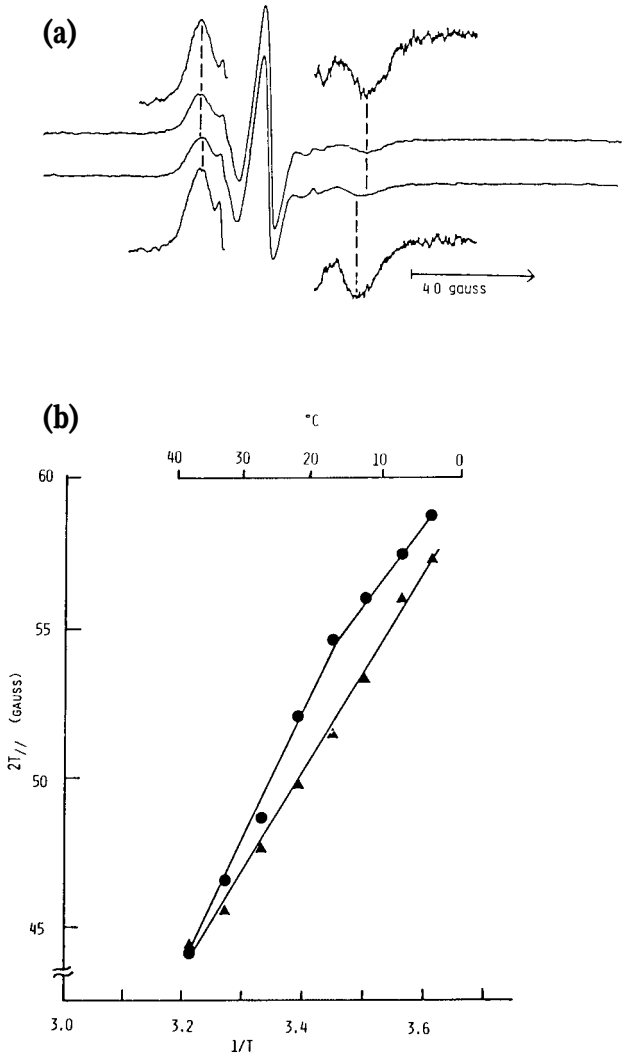

Fig. 3. (a) Electron spin resonance spectra of I (5, 10)-labeled erythrocytes.
(upper spectra) control, (lower spectra) LCAT deficiency subject.
(b) Temperature dependence of the overall splitting (gauss).
(●) control, (▲) LCAT deficiency subject

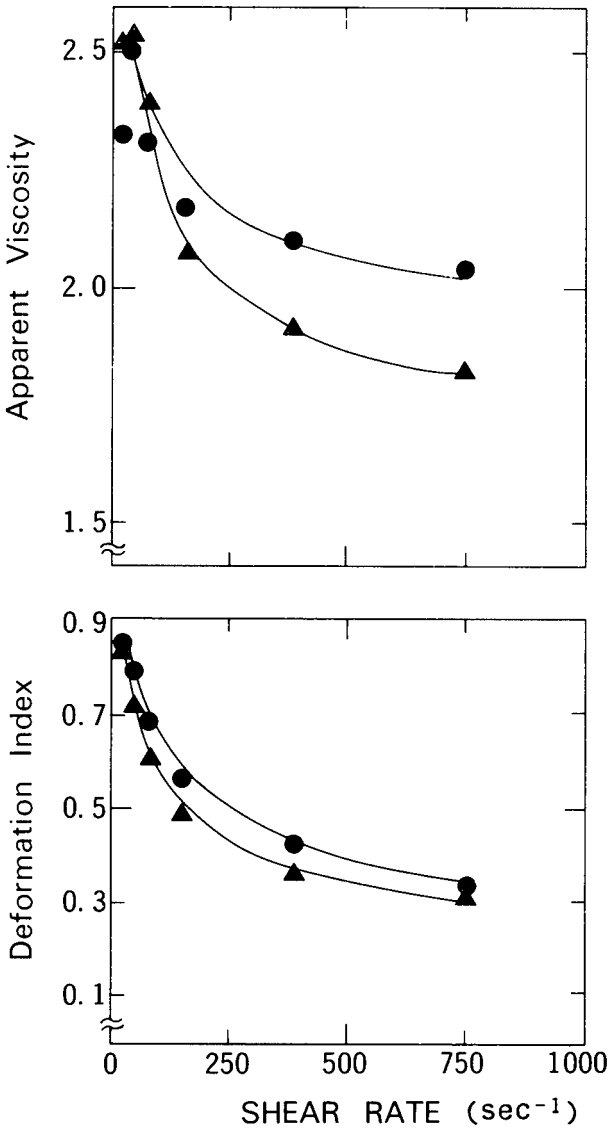

Fig. 4. Viscosity (upper panel) and deformability of erythrocytes (lower panel) under various shear rates. Viscosity and deformability of patient's erythrocytes were decreased compared to those of a normal subject.
(●) control, (▲) LCAT deficient subject

brane was one of the factors which increased the osmotic fragility. However, in this patient, another parameter of osmotic fragility, H25-75, was increased 1.7 fold compared with that of the normal subject. If the increased osmotic resistance was due to the increased C/P ratio of the membrane, the other parameter would decrease followed by increased free cholesterol in the membrane (9, 13, 16). Thus, the change of osmotic fragility in the patient might suggest other factors such as the increased surface area of the membrane and/or mean corpuscular volume due to lipid abnormality in the membrane. It has been reported that erythrocytes with LCAT deficiency possess a short life-span *in vivo*. It had been accepted that erythrocyte deformability was one of the major factors in the destruction of circulation. We, as well as other investigators, reported previously that deformability, membrane fluidity and cholesterol content are closely related : that is, increased cholesterol concentration in the erythrocyte membrane decreases deformability due to the decreased membrane fluidity of the cells (16). This patient's cells, however, possessed decreased deformability in spite of elevated membrane fluidity. In 1997, Abugo *et al*. showed that geometric properties such as mean corpuscular volume and excess surface area were related to the deterioration of the red cell mechanical properties in capillary flow (17). In this case, the amount of membrane lipids (free cholesterol and total phosphpolipids) accumulated to 127% due to

the deterioration of lipid equilibrium. Therefore, the abnormal shape of the patient's erythrocytes was related to the depletion of the partial free cholesterol by LCAT from the patient's membrane followed by decreased abnormal shapes *in vitro* in Table 4.

The total lipid amount of free cholesterol and phosphpolipids increased by 27% associated with the completely different phospholipid compositions. The increased phosphatidylchone (158%) and decreased phosphatidylethanolamine (42%) in the membrane might contribute to the increased fluidity of the patient's membrane in spite of the slight increase in C/P ratio.

Recently, Jain *et al*. (18) indicated that a patient's erythrocytes were more unstable mechanically in hypotonic medium due to membrane fragmentation of the cells, and were more susceptible to peroxidant stress due to the increased membrane level of polyunsaturated fatty acid. In our case, the fatty acid compositions of phospholipids in the membrane did not change significantly (data not shown). On the other hand, Flamm K and Schacter D (19) showed that erythrocyte membranes with betalipoprotein deficiency and enriched membrane cholesterol decreased the fluidity of the outer but not the inner hemileaflet. It is interesting that erythrocyte membranes (acanthocytes) with betalipoprotein deficiency have increased cholesterol and phosphatidylethanolamine, but decreased phosphatidylchone, which is opposite to those changes in LCAT deficiency.

Concerning the asymmetric distribution of membrane phospholipids as a sort of lipid equilibrium, membrane proteins such as spectrin play an important role in the maintenance of membrane lipid asymmetry in erythrocytes (20, 21). The membrane proteins of our patient did not reveal any abnormalities (data not shown).

These results suggest that erythrocytes with LCAT deficiency may have an abnormal intramembrane distribution of lipids, and abnormal properties such as deformability, membrane fluidity, mean corpuscular volume and the shape of the cells may attribute to the abnormal distribution of membrane lipids.

This was supported by the fact that we are able to prevent the increase of transformed cells by means of cholesterol depletion from the membrane (Table 4). As a preliminary experiment for confirmation, intact erythrocyte membranes were exposed to cholesterol oxidase from outside the membrane. Although all the membrane cholesterol was oxidized, cholesterol in the patient's membrane exhibited less reactivity (ca. 4 fold) despite the total amount of cholesterol being richer than in normal cells. These results indicate that anemia, abnormal shape and the increased mean corpuscular volume of this patient's erythrocytes may be related to membrane instability owing to the abnormal distribution of membrane lipids such as cholesterol, phosphatidylcholine and phosphatidylethanolamine.

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