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Changes in Shape and Osmotic Resistance of Human Erythrocytes resulted from Changes in the Lysolecithin Content of the Membranes[1]

Takashi Sato[2] and Tatsuzo Fujii[2a]

Faculty of Pharmacy, Meijo University[2]

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Incubation of human erythrocytes in lysolecithin-containing medium, prepared by an addition of purified lysolecithin to blood plasma or to phosphate-buffered saline, induced the increases in lysolecithin content of the erythrocyte membranes as well as morphological changes of the cells from normal biconcave disc to crenated or spherical forms. The erythrocytes with slight excess of lysolecithin incorporated, which take shapes of crenated disc or crenated sphere, rather have increased osmotic resistance, whereas those with much more lysolecithin, taking almost spherical form, show reduced osmotic resistance. After washing these erythrocytes with heated normal plasma to remove excess lysolecithin from the membrane, the increased osmotic resistance observed in the former erythrocyte preparation was restored to the normal level, whereas the decreased resistance in the latter cell preparation was never reversed, in spite of the restoration to the normal morphological shape.

As to the effects of lysolecithin in sub-hemolytic concentration on human erythrocytes, it was already reported by Klibansky and De Vries[3] and by Sato[4] that when human erythrocytes were incubated with lysolecithin-enriched plasma prepared by previous enzymatic conversion of the plasma lecithin to lysolecithin, the shape and the osmotic resistance of the erythrocytes were remarkably changed as the amounts of the lysophospholipid bound to the membrane were increased. In these reports, lysolecithin-enriched plasma was prepared by the action of added snake venom phospholipase A[3,4] or of plasma lecithin-cholesterol acyltransferase.[4] These enzymes, however, use lecithin or both lecithin and cholesterol in plasma lipoprotein as their substrate or substrates and subsequently the amount of plasma lecithin (and cholesterol) should be decreased markedly with the increase in plasma lysolecithin. It is not quite improbable, therefore, that the above-mentioned changes in erythrocyte, considered to be resulted from increased lysolecithin content, might be caused also by the decrease in lecithin or cholesterol content of the erythrocyte membrane, or by a direct action of the added phospholipase A remaining in the plasma.

In the present report, in order to confirm the effect to be really caused by lysolecithin, human erythrocytes were incubated with lysolecithin-enriched plasma which had been prepared by the direct addition of pure lysolecithin into normal plasma, or incubated with lysolecithin dispersion in physiological saline, and incorporation of lysolecithin into the erythrocyte membrane, changes in the shape and the osmotic resistance of the erythrocyte were similarly investigated.

Experimental

Preparation of Washed Erythrocyte Suspension——Fresh-drawn blood was centrifuged at $900 \times g$ for 15 minutes. The supernatant plasma was used as the medium into which pure lysolecithin be added. After

1) Part of this work was presented at the 92nd Annual Meeting of the Japanese Pharmaceutical Society, Osaka, April 1972, and also at the 45th Annual Meeting of the Japanese Biochemical Society, Tokyo, November 1972.

2) Location: *Yagoto Urayama 15, Tenpakucho, Showaku, Nagoya*; *a*) Present address: *Kyoto College of Pharmacy, Yamashina, Higashiyamaku, Kyoto.*

3) C. Klibansky and A. De Vries, *Biochim. Biophys. Acta*, **70**, 176 (1963).

4) T. Sato, *Chem. Pharm. Bull.* (Tokyo), **21**, 176 (1973).

removal of the buffy layer, the residual erythrocytes were washed three times with 0.15M NaCl and resuspended in the saline.

Preparation of Lysolecithin-Enriched Medium——Lysolecithin was prepared from egg yolk lecithin by the action of snake venom phospholipase A and purified by means of silicic acid column chromatography. This lysolecithin preparation, detected as one spot on two-dimensional thin-layer chromatogram, was directly added and dissolved in plasma or phosphate buffered saline (154 mM NaCl in 10 mM phosphate buffer, pH 7.4; PBS). In the case of PBS, lysolecithin added was dispersed by sonication.

Attachment and Detachment of Lysolecithin to and from the Erythrocyte Membrane——Washed erythrocytes were incubated at 37° for 10 minutes with lysolecithin-enriched plasma or PBS prepared as above, in 40 or 20% hematocrit proportion. An aliquot of the erythrocyte suspension was then centrifuged at $900 \times g$ for 15 minutes and washed twice with saline. The residual portion of the erythrocytes was allowed to stand at room temperature for 5 minutes with heated plasma (lecithin-cholesterol acyltransferase inactivated), and then centrifuged to separate the supernatant plasma. This process was further repeated once. By these procedures, lysolecithin once attached to the erythrocyte membrane was removed into the plasma. The residual erythrocytes were washed twice with saline.

Lipid Extraction and Quantification of Phospholipids——Lipids were extracted from erythrocyte stroma with chloroform–methanol mixture according to the method of Ways and Hanahan.[5] Quantitative estimation of phospholipids was carried out according to the method of Burger, Fujii and Hanahan[6] by means of thin-layer chromatography. Phospholipid phosphorus was assayed by Bartlett's method.[7]

Scanning Electron Microscopic Observations and Test for Osmotic Resistance of Erythrocytes——These were carried out as reported in our previous paper.[4]

Result and Discussion

Table I shows increases in the amount of lysolecithin in erythrocyte membrane when normal erythrocytes were incubated with lysolecithin-enriched plasma. The increases depend on the lysolecithin concentration of the surrounding medium. At a concentration of 11.1 or 14.2×10^{-7} moles of lysolecithin per 1 ml of plasma, the incorporation reached practically the maximum value and the lysolecithin content of the membrane was about 4 times as high as that of the normal erythrocytes. Only slight hemolysis (about 1—2%) was observed at such a lysolecithin concentration in medium. Below this concentration, practically no hemolysis was observed.

Table I. Changes in Lysolecithin Content of Erythrocyte Membrane after Incubation (37°, 10 min) with Lysolecithin-Enriched Medium

Medium	Lysolecithin content of medium		Lysolecithin content of erythrocyte	
	$\times 10^{-7}$ mole per ml medium	$\times 10^{-7}$ mole per ml cell	$\times 10^{-7}$ mole per ml cell	(increase over normal value)
Heated plasma[a]	2.1 (normal)	3.15	1.01	
	4.7	7.05	1.82	(+0.81)
	11.1	16.7	4.16	(+3.15)
	14.2	21.3	4.32	(+3.31)
PBS[b]	0.1	0.40	1.91	(+0.90)
	1.0	4.00	3.59	(+2.58)

a) Normal plasma heated at 56° for 30 minutes, to which erythrocytes were suspended in 40% hematocrit concentration.
b) Isotonic phosphate-buffered saline (NaCl), pH 7.4, to which erythrocytes were suspended in 20% hematocrit concentration

In the case of incubation in isotonic phosphate-buffered saline (PBS), the maximal incorporation of lysolecithin was observed at 10^{-7} mole of lysolecithin per ml of the medium. At this concentration, hemolysis was about 4% and at the higher concentrations hemolysis was very marked (about 65% at 5×10^{-7} mole per ml medium).

5) P. Ways and D.J. Hanahan, *J. Lipid Res.*, **5**, 318 (1964).
6) S.P. Burger, T. Fujii and D.J. Hanahan, *Biochemistry*, **7**, 3682 (1968).
7) G.R. Bartlett, *J. Biol. Chem.*, **234**, 466 (1959).

The lysolecithin concentrations in the media, employed in these two types of incorporation experiments, are not directly comparable, because the concentration of the erythrocytes suspended in the medium (hematocrit value) is different, namely 40% in plasma and 20% in PBS. Therefore, amounts of the medium lysolecithin per ml of erythrocytes contained in the medium were calculated and shown in Table I. Thus, it is evident that the amount of lysolecithin in medium to bring about the maximal incorporation into erythrocyte membrane is about 16.7×10^{-7} moles per ml of the cells in plasma and 3.0×10^{-7} moles per ml of the cells in PBS. Such a difference may be due to the lysolecithin-binding capacity of the plasma proteins which reduced the plasma concentration of free lysolecithin which can actually bind to erythrocyte membrane.

It was then demonstrated that with such amount of extra lysolecithin incorporated, no quantitative alteration of the membrane phospholipids other than lysolecithin was caused, as shown in Table II. The cholesterol content of the membrane was also not altered.

Table II. Lipid Contents of the Treated[a] and Untreated Erythrocytes

Lipid classes	Lipid content ($\times10^{-7}$ mole/ml of packed cells)		
	Normal cell	Lysolecithin-enriched cell	Lysolecithin-enriched cell, after washing with normal heated plasma
Total phospholipid	39.4	38.0	39.4
Phosphatidylethanolamine	10.9	10.0	10.7
Lecithin	9.76	9.09	10.4
Sphingomyelin	10.8	8.74	9.76
Lysolecithin	1.30	3.59	1.53
Others	6.49	6.60	7.04
Cholesterol	31.3	28.4	34.8

a) by treated with PBS containing 1.0×10^{-7} mole per ml medium of lysolecithin (as reported in tne lowest line of Table I)

In order to detect changes in osmotic resistance of the erythrocytes due to the increase in the lysolecithin content, hemolysis of the lysolecithin-enriched erythrocytes in hypotonic medium was measured as shown in Table III and IV.

Table III. Relative Hemolysis of Erythrocytes in Hypotonic Buffered-Saline[a] after Incubation with Plasma of Varied Lysolecithin Concentrations

Medium lysolecithin in plasma		Erythrocyte			
		Lysolecithin-enriched cell		Lysolecithin-enriched cell, after washing with heated normal plasma	
$\times10^{-7}$ mole per ml medium	$\times10^{-7}$ mole per ml cell	Relative hemolysis[b] (%)	Shape[c]	Relative hemolysis[b] (%)	Shape[c]
2.1 (normal)	3.15	50.6	biconcave disc[d]		
3.5	5.25	31.8	biconcave disc +crenated disc[d]	50.3	biconcave disc
4.8	7.20	34.6	crenated disc +crenated sphere[d]	52.3	biconcave disc
6.0	9.00	36.7	crenated sphere[d]		
7.3	11.0	64.1	crenated sphere		
7.9	11.9	76.4	crenated sphere	78	
11.1	16.7	92	smooth sphere[d]	98	biconcave disc
14.2	21.3	94	smooth sphere		

a) 55 mM NaCl in 10 mM phosphate buffer, pH 7.4
b) Hemolysis in 20 mM NaCl-10 mM phosphate buffer being taken as 100%.
c) observed under scanning electron microscope
d) The electron micrographs shown in Fig. 1.

Table IV. Relative Hemolysis of Erythrocytes in Hypotonic Buffered-Saline after Incubation with PBS of Varied Lysolecithin Concentrations

Lysolecithin in medium		Lysolecithin-enriched erythrocyte	
$\times 10^{-8}$ mole per ml medium	$\times 10^{-7}$ mole per ml cell	Relative hemolysis (%)	Shape
0	0	62.7	biconcave disc
0.2	0.08	59.1	biconcave disc+ crenated disc
0.5	0.20	56.4	biconcave disc+ crenated disc
0.8	0.32	52.9	biconcave disc+ crenated disc
1.0	0.40	48.2	crenated disc
1.5	0.60	58.7	crenated disc+ crenated sphere
2.0	0.80	61.5	crenated disc+ crenated sphere
5.0	2.0	62.6	crenated sphere
7.0	2.8	76.2	crenated sphere
10.0	4.0	95.9	smooth sphere

It was disclosed that the erythrocytes which were incubated with a low concentration of lysolecithin in either of the media (plasma and PBS) were made osmotically resistant by the slight excess lysolecithin bound to the membrane. Such protective effect of the membrane lysolecithin against hypotonic hemolysis was observed most significantly at a concentration of 3.5×10^{-7} moles per ml of plasma and 10^{-8} moles per ml of PBS. At these concentrations, the lysolecithin content of erythrocyte membrane was approximately twice as much as the normal (Table I). Such stabilizing effect of erythrocyte membrane, induced by lysolecithin incorporation, was previously reported by Seeman[8] and our present results are in good agreement with his results. Observation of morphological changes of the lysolecithin-enriched erythrocyte at relatively low medium lysolecithin concentration, showed that biconcave discs of normal cell were deformed to crenated disc or crenated sphere. In higher concentration, the projections on the erythrocyte membrane surface become shrunken and spherical cells appear. Scanning electron micrographs of the erythrocytes undergone such succession of changes are presented in Fig. 1.

As already reported,[4] such morphological changes as well as the changes in membrane lysolecithin content are completely reversed by washing the erythrocytes with heated normal plasma to remove the excess lysolecithin bound to the membrane, whereas the reduced osmotic resistance of the erythrocytes with very high lysolecithin content can not be reversed. It now becomes evident, however, that the osmotic resistance of erythrocytes once raised with slight excess of the membrane lysolecithin is then reversibly restored to the normal level by removing the excess lysolecithin. As shown in Table III erythrocytes made osmotically more resistant regained a normal osmotic property after washing them with heated normal plasma.

The above-reported results are summarized in Fig. 2. It discloses that changes in membrane properties, represented by its osmotic resistance, which were caused by excess membrane-bound lysolecithin, are closely correlated with the morphological changes of the membrane. In the case of the erythrocytes suspended in lysolecithin-enriched plasma in 40% hematocrit

8) P. Seeman, *Biochem. Pharmacol.*, **15**, 1767 (1966).

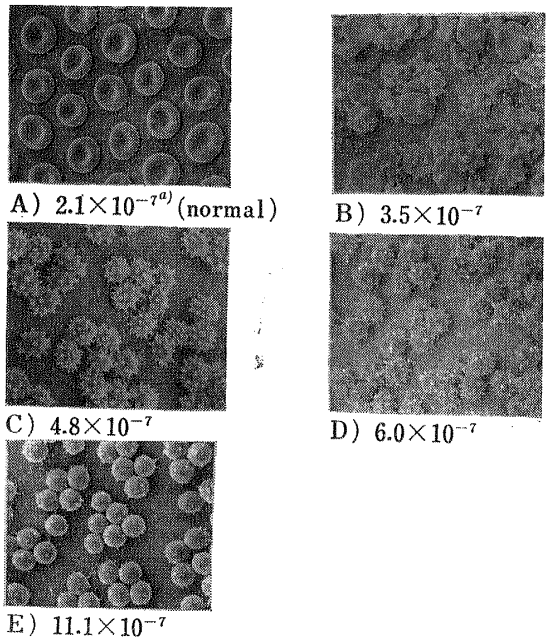

Fig. 1. Changes in Erythrocyte Morphology Depending on the Plasma Lysolecithin Concentration as revealed by Scanning Electron Microscopy

a) The figures indicate plasma lysolecithin concentration as mole per ml of medium.

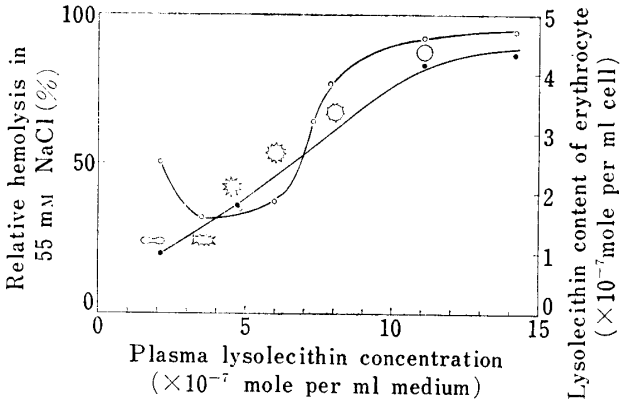

Fig. 2. Correlation among the Changes in Morphology, Osmotic Resistance and Lysolecithin Content of Human Erythrocytes

—●—: lysolecithin content
—○—: relative hemolysis

proportion, stabilizing effect of the lysophospholipid on the erythrocyte membrane is exerted in a range of lysolecithin concentration of about 3—6×10^{-7} mole per ml of the medium which produces crenated disc or crenated sphere forms of the cells. Over a concentration of about 7×10^{-7} mole per ml medium, where spherical forms with short projections or almost smooth spheres occur, membrane-labilizing effect now appears and progressively becomes pronounced with the increasing lysolecithin concentration. Then, hemolysis ensues.

In the course of such morphological changes of erythrocytes, no change in their cell volumes was detected, as determined by hematocrit estimation. It is suggested, therefore, that the changes from normal biconcave disc to spherical forms must bring about decrease in the membrane surface area which may be caused by contraction of the membrane as the result of excess binding of lysolecithin. Removal of such excess lysolecithin might give relaxation of the membrane and the restoration of the surface area. Consequently the spherical forms returned to the normal form.

The results obtained by the present study are in agreement with the data previously reported by one of us[4] and support the view that the increased lysolecithin content of erythrocyte membrane is really responsible for the changes in the shapes and osmotic resistance of the membrane.