CHANGES IN MEMBRANE FLUIDITY OF ERYTHROCYTES DURING CELL MATURATION

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SUMMARY

The measurements of the fluorescence polarization of perylene embedded in erythrocyte membranes were carried out with normal and reticulocyte-rich blood, and the microviscosity of erythrocyte membranes was calculated from the polarization degree. In intact cells, reticulocyte membranes had a significantly lower microviscosity than normal erythrocyte membranes, while in ghosts no significant difference in membrane microviscosity was observed between reticulocytes and mature erythrocytes.

INTRODUCTION

It is well known that phenylhydrazine treatment induces severe anemia in mammals followed by the release of reticulocytes from the bone marrow (1-3). By using this procedure, the reticulocyte-rich blood can easily be obtained. The reticulocyte is considered to be released prematurely from the bone marrow (4) and has a volume of almost double that of the mature cell. It undergoes normal maturation during circulation (5). Therefore, the system has been used in the various studies on cell maturation (6-10).

Since the fluidity of plasma membranes in chick embryo heart has been shown to change during development (11), we intended to investigate the change in the fluidity of erythrocyte membranes during maturation. In the present study, the membrane microviscosity of erythrocytes of phenylhydrazine-treated rabbits was measured with fluorescence polarization technique (12-16). Perylene was adopted as a fluorescent probe.

MATERIALS AND METHODS

Albino rabbits weighing approximately 2 kg were made anemic by the injection of phenylhydrazine as described by Koch et al. (7). Blood samples were

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collected into heparinized glass tubes (100 units/ml of blood) by ear vein puncture. Erythrocytes were washed 3 times with cold isotonic saline. The buffy coat was carefully removed by aspiration. The cells were resuspended in a medium containing 0.15 M NaCl, 10 mM Tris-HCl (pH 7.4) and 10 mM glucose (1×10^7 cells/ml). Erythrocytes were counted in a Toa microcell counter type CC-1002, and the mean corpuscular volume was determined in the same instrument equipped with a Toa hematocounter type HT-310. Reticulocytes were counted by the standard hematological method using Brilliant Cresyl Blue. At least 1,000 cells were counted per sample.

Thirty µl of ethanol solution of perylene (4×10^{-4} M) was introduced into 4 ml of the cell suspension, and the medium was incubated at 37°C for 30 min. The labelled cells were washed twice with cold isotonic saline to remove excess perylene, resuspended in a medium containing 0.15 M NaCl and 10 mM Tris-HCl (pH 7.4) to be $3\text{-}5 \times 10^6$ cells/ml and subjected to fluorescence measurement. To obtain the lysed cell preparation (ghosts), the labelled cells were first lysed in 10 mM Tris-HCl (pH 7.4) and, after 5 min of incubation at 0°C, the medium was made isotonic by the addition of concentrated NaCl solution. In some experiments, ghosts were washed twice with 10 mM Tris-HCl (pH 7.4) and resuspended in 3 ml of a medium containing 0.15 M NaCl and 10 mM Tris-HCl (pH 7.4). This preparation was used as hemoglobin-free ghosts.

Erythrocytes of reticulocyte-rich blood were separated into age groups according to Walls et al. (17) except that the specific gravities of three albumin solutions (from bovine serum, Sigma) were adjusted to 1.085, 1.090 and 1.096. After centrifugation, the cells separated into four bands including the bottom sediment. The top and the bottom band were taken, washed 4 times with a medium containing 0.15 M NaCl and 10 mM Tris-HCl (pH 7.4) and used for fluorescence analysis. The top band corresponded to the younger cells, whereas the bottom band to the older ones.

The degree of polarization was measured at 25°C with a Shimadzu spectrofluorophotometer RF-502 equipped with Polacoat polarized filters as described previously (18). Perylene was excited at 435 nm and the emission was monitored at 475 nm. The membrane microviscosity, $\bar{\eta}$, was calculated from the following formula: $r_0/r = 1 + kT\tau/V(r)\bar{\eta}$, where r is a degree of fluorescence anisotropy obtained from polarization degree, r_0 a limiting anisotropy for perylene (0.370) (Ref. 19), k a Boltzman's constant and V(r) the effective rotational molecular volume (12). The values of k/V(r) were taken from the data (19) obtained with reference oil. T is the absolute temperature and τ the mean fluorescence lifetime. Fluorescence lifetime was measured with a monophoton counting technique.

RESULTS AND DISCUSSION

Polarization degrees of perylene fluorescence embedded in erythrocytes obtained from normal and reticulocyte-rich blood are shown in Table 1. The polarization degree of perylene fluorescence in reticulocyte-rich cells is significantly lower than that in normal cells, while in lysed cells or in hemoglobin-free ghosts the difference in polarization degree between the two is not significant. Since it has been shown that the hemoglobin-free ghosts of reticulocyte-rich cells contained a minimal amount of ribosomes and no mitochondria (7), the present data indicate that the possible contribution of the

Table 1 Membrane microviscosity of erythrocytes obtained from normal and reticulocyte-rich blood

Sample	Polarization degree	Membrane microviscosity $\bar{\eta}$ (poise)
Normal blood*		
Intact cells	0.141	6.1
Lysed cells	0.123	4.6
Hemoglobin-free ghosts	0.123	4.6
Reticulocyte-rich blood**		
Intact cells	0.105	3.5
Lysed cells	0.123	4.6
Hemoglobin-free ghosts	0.126	4.8

* This sample contained 3.2% reticulocytes.
** This sample was taken from the rabbit 3 days after the daily injections of phenylhydrazine (6 mg/kg) for 5 days. It contained 55.5% reticulocytes. Data represent the mean of triplicate measurements.

dye associated with the residual intracellular membrane system of reticulocyte to the value of membrane microviscosity is negligible.

Fluorescence intensity was weak in the presence of hemoglobin. This is probably due to the reabsorption of the fluorescence by hemoglobin which has strong absorbance in the wavelength region of the measurement. Because of the weak fluorescence, the scattering of the exciting light was carefully corrected using an unlabelled sample, as was done by Aloni et al. (14).

Membrane microviscosity was calculated from the polarization degree of the fluorescence and the excited state lifetime of the perylene. The lifetime of perylene embedded in hemoglobin-free ghosts was measured to be 9.2 nsec. The same value was also obtained with perylene embedded in liposomes prepared from the lipids extracted from the ghosts (20). As to the intact cells, however, we could not obtain its reliable value owing to the weakness of the fluorescence mentioned above. Accordingly, we assumed that the lifetime of the dye embedded in the intact cells is the same as that in the ghosts, since the reported result (21) indicates that the excited state lifetime of perylene in the membranes of polymorphonuclear leucocyte is not different from that in the liposomes prepared from their lipids.

Table 2 Membrane microviscosity of erythrocytes obtained from phenylhydrazine-treated rabbit at various stages of anemia

Sample	Mean corpuscular volume (μ^3)	Reticulocyte (%)	Membrane microviscosity $\bar{\eta}$ (poise)	
			Intact cells	Ghosts
Control	60.7	2.3	5.81 ± 0.65 (6)	4.50 ± 0.13 (6)
Day 1	55.8	23.5	4.23 ± 0.51 (4)**	4.49 ± 0.27 (4)
Day 3	101.0	63.2	3.44 ± 0.15 (6)**	4.57 ± 0.15 (6)
Day 6	77.4	30.0	4.10 ± 0.13 (5)**	4.51 ± 0.25 (5)
Day 11	70.5	13.9	4.65 ± 0.21 (5)*	4.60 ± 0.33 (5)
Day 42	60.0	4.1	5.95 ± 0.28 (5)	4.59 ± 0.39 (5)

Day 1 represents the sample taken one day after the last injection of phenylhydrazine, Day 3 that taken 3 days after the last injection and so on. The means ± S.D. of membrane microviscosity are shown. The number of experiments is given in parenthesis.

Table 2 shows the membrane microviscosity of the intact cells and the ghosts containing various amounts of reticulocytes. Blood was taken from a phenylhydrazine-treated rabbit at the various stages of anemia. Hematological data obtained with phenylhydrazine-treated rabbit are similar to those reported by Seno (2). The membrane microviscosity of the intact cells showed a minimum in Day 3 sample containing a maximal amount of reticulocytes, while in the ghosts, the membrane microviscosity was not proportional to the amount of reticulocytes contained. When phenylhydrazine was added to the suspension of the mature cells up to 5×10^{-5} M, the membrane microviscosity did not change significantly. Therefore, the direct effect of phenylhydrazine on membrane microviscosity seems to be negligible.

Table 3 shows the membrane microviscosity of the reticulocytes in comparison with that of the mature cells. The top fraction containing a larger amount of reticulocytes (85.2%) has a lower membrane microviscosity (2.9 poise) than the unseparated sample (3.5 poise), while the membrane microviscosity of the bottom fraction containing a minimal amount of reticulocytes (3.1%) is much higher (5.0 poise) than that of the unseparated sample. These data clearly indicate that the low value of the membrane microviscosity of the intact cells

Table 3 Membrane microviscosity of young and old erythrocytes separated from reticulocyte-rich blood

Sample	Mean corpuscular volume (μ^3)	Reticulocyte (%)	Membrane microviscosity $\bar{\eta}$ (poise)	
			Intact cells	Ghosts
Unseparated	97.6	65.9	3.5	4.6
Top	107.0	85.2	2.9	4.5
Bottom	38.5*	3.1	5.0	4.2

Half milliliter of washed erythrocytes from one of Day 3 samples was centrifuged on 3 layers of specific gravity (top to bottom) 1.085, 1.090 and 1.096 in 12 ml polyallomer tube at 160,000 × g for 1 h. After centrifugation, the top and the bottom fraction were taken, washed 4 times with a medium containing 0.15 M NaCl and 10 mM Tris-HCl (pH 7.4) and were subjected to the hematological and the fluorescence analysis. Data represent the means of triplicate measurements.

* This small volume might be ascribed to the shrinkage of the cell caused by phenylhydrazine.

in Day 3 sample is due to the increase of reticulocytes in the sample.

The above results indicate that the fluidity of the reticulocyte membranes decreases with cell maturation. Since the change in membrane fluidity was not observed in the ghosts during cell maturation (see Tables 2 and 3), it is suggested that some fine structure of the cell membranes, which can be easily damaged by hypotonic lysis, may regulate the membrane fluidity of erythrocytes.

REFERENCES

1. Archdeacon, J. W. and Rohrs, H. C. (1962) Am. J. Physiol., 203, 283-285.
2. Seno, S. (1964) Acta Haematol., 31, 129-136.
3. Simpson, C. F. and Kling, J. M. (1968) J. Cell Biol., 36, 103-109.
4. Seno, S., Miyahara, M., Asakura, H., Ochi, O., Matsuoka, K. and Toyama, T. Blood, 24, 582-593.
5. Ganzoni, A., Hillman, R. S. and Finch, C. A. (1969) Br. J. Haematol, 16, 119-135.
6. Lodish, H. (1973) Proc. Natl. Acad. Sci. USA, 70, 1526-1530.
7. Koch, P. A., Gardner, F. H. and Carter, Jr. J. R. (1973) Biochem. Biophys. Res. Commun., 54, 1296-1299.
8. Babu, C. R., Azhar, S. and Murti, C. R. K. (1975) Medical Biol., 53, 148-155.
9. Gauger, D., Kaiser, G., Quiring, K. and Palm, D. (1975) Naunyn-Schmiedeberg's Arch. Pharmacol., 289, 379-398.
10. Bartlett, G. R. (1976) Biochem. Biophys. Res. Commun., 70, 1055-1062.
11. Kutchai, H., Barenholz, Y., Ross, T. F. and Wermer, D. E. (1976) Biochim. Biophys. Acta, 436, 101-112.

12. Shinitzky, M., Dianoux, A. C., Gitler, C. and Weber, G. (1971) Biochemistry, 10, 2106-2113.
13. Rudy, B. and Gitler, C. (1972) Biochim. Biophys. Acta, 288, 231-236.
14. Aloni, B., Shinitzky, M. and Livne, A. (1974) Biochim. Biophys. Acta, 348, 438-441.
15. Sha'afi, R. I., Rodan, S. B., Hintz, R. L., Fernandez, S. M. and Rodan, G. A. (1975) Nature, 254, 525-526.
16. Feinstein, M. B., Fernandez, S. M. and Sha'afi, R. I. (1975) Biochim. Biophys. Acta, 413, 354-370.
17. Walls, R., Kumar, K. S. and Hochstein, P. (1976) Arch. Biochem. Biophys., 174, 463-468.
18. Yagi, K., Tanaka, F. and Ohishi, N. (1975) J. Biochem., 77, 463-468.
19. Cogan, U., Shinitzky, M., Weber, G. and Nishida, T. (1973) Biochemistry, 12, 521-528.
20. Tanaka, F., Hagihara, M., Katsumata, Y., Ohara, T., Tanaka, J. and Yagi, K. manuscript in preparation.
21. Berlin, R. D. and Fera, J. P. (1977) Proc. Natl. Acad. Sci. USA, 74, 1072-1076.