Journal of Supramolecular Structure 9:453–458 (1978)
Normal and Abnormal Red Cell Membranes 305–310

Bilayer Balance and Regulation of Red Cell Shape Changes

Narla Mohandas, Alfred C. Greenquist, and Stephen B. Shohet

Departments of Laboratory Medicine and Medicine, the Division of Hematology (N.M., A.C.G., S.B.S.); and the Cancer Research Institute, University of California at San Francisco (N.M., S.B.S.), San Francisco, California 94143

Discocytic human red cells undergo discocyte-echinocyte and discocyte-stomatocyte transformations under the action of a wide variety of lipid-soluble anionic and cationic agents respectively. These shape transformations are explained by the bilayer couple hypothesis of Sheetz and Singer to be the result of preferential distribution of the anionic agents in the outer half of the bilayer and the cationic agents in the inner half of the bilayer. We demonstrate that echinocytogenic effects indeed occur when the naturally occurring phospholipid lysophosphatidylcholine (LPC) is localized in the outer half of the bilayer, and stomatocytogenic effects occur when LPC is in the inner half. However, in contrast to the bilayer couple hypothesis, our results show that simple equivalent membrane surface area expansion on each layer is insufficient to maintain the discocytic shape and there exists a differential concentration effect of LPC on the two halves of the bilayer.

Key words: red cell, membranes, bilayer, morphology, echinocyte, stomatocyte, phospholipid, lysolecithin

Excellent evidence now exists that the protein and lipid components of the biologic membrane are asymmetrically distributed within the two monolayers [1, 2]. In human red cells phosphatidylcholine (PC) and sphingomyelin lie mainly in the external monolayer, while the aminophosphatides (phosphatidylethanolamine and phosphatidylserine) are for the most part in the internal or cytoplasmic monolayer [2–4]. Sheetz and Singer have made the attractive suggestion that this asymmetrical distribution of phospholipids produces the separation of electrostatic charge, leading to differences in the transmembrane localization of compounds that induce shape changes in red cells [5, 6]. They proposed that, at equilibrium, anionic and cationic drugs are preferentially distributed in the outer and inner half of the lipid bilayer respectively. According to this hypothesis, anionic drugs

Received March 23, 1978; accepted August 8, 1978.

0091-7419/78/0903-0453$01.70 © 1978 Alan R. Liss, Inc.

would expand the area of the outer surface of the membrane relative to the inner surface and cause the cells to become echinocytic (crenated), and cationic compounds would expand the inner surface area relative to the outer and cause the cells to become stomatacytic (cup shaped) [7].

In this paper we show that varying the leaflet location of the natural membrane phospholipid lysophosphatidylcholine (LPC) indeed induces different red cell shape changes. However, the changes occur with differing sensitivity to LPC in the inner and outer halves of the bilayer, and indicate that simple membrane leaflet expansion cannot explain the shape change effects of LPC.

MATERIALS AND METHODS

Blood was drawn from healthy human volunteers into heparinized vacutainers, and the red cells were washed twice in saline. Washed red cells were incubated at a 5% hematocrit in phosphate-buffered saline (PBS) (pH 7.4, 290 mOsm) with glucose, 0.5% human se serum albumin, and 70 μmoles/liter of the echinocytic agent, LPC. Lysopalmitoyl phosphatidylcholine (Palmityl-^{14}C) was used as a tracer to determine membrane concentration of LPC by radioactive counting.

The incubations were carried out at 37°C. Five min after the start of incubation and at 2.5-h subsequent intervals, two 0.25-ml aliquots of the red cell suspension were taken, one washed four times (15 ml wash solution) with PBS and the other four times with 1% bovine serum albumin in PBS. After the final wash the red cells were dissolved in 0.75 ml Protosol (New England Nuclear), decolorized with H_2O_2, and subsequently counted in a liquid scintillation counter using Aquasol as counting fluid.

To determine the extent of acylation of LPC into phosphatidylcholine, aliquots of the incubating red cell suspension were also taken at the same time intervals to determine the percentage of total radioactivity in the LPC and PC fractions after albumin and saline washings by thin-layer chromatography [8]. Red cell morphology was monitored by phase contrast microscopy and scanning electron microscopy [9].

RESULTS

LPC Content

Red cell membrane localization of ^{14}C-LPC at regular incubation intervals is shown in Figure 1. LPC was incorporated into the red cell membrane as evidenced in 5 min by the rapid rise of ^{14}C counts in saline-washed red cells. Subsequently, LPC content of the red cells increased linearly with time in both the saline- and albumin-washed cells (Fig. 1). Because of the high affinity of albumin for LPC and the large amounts of albumin used in washing, it is reasonable to assume that the LPC of red cells after saline washing represented the total LPC in both halves of the bilayer, while the LPC after albumin washing represented the inaccessible LPC present in only the inner half of the bilayer. The outer leaflet LPC was calculated by subtracting the inner leaflet content from the total.

This proposed localization of residual LPC in the inner monolayer of albumin-washed cells was confirmed by our experiments with resealed and unsealed ghosts made from red cells after 7.5 hours of incubation and subsequent albumin washing. None of the residual LPC could be removed from the resealed ghosts by further albumin washes, whereas 95% of the LPC was removed by albumin washing of the unsealed ghosts. An increase in the ^{14}C-labeled phosphatidylcholine content of the red cell also occurred as a result of

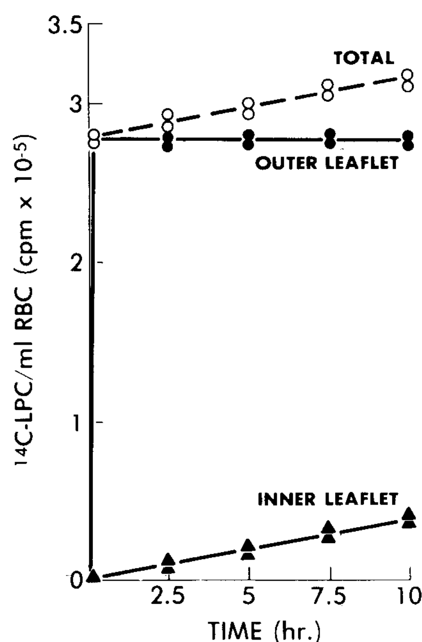
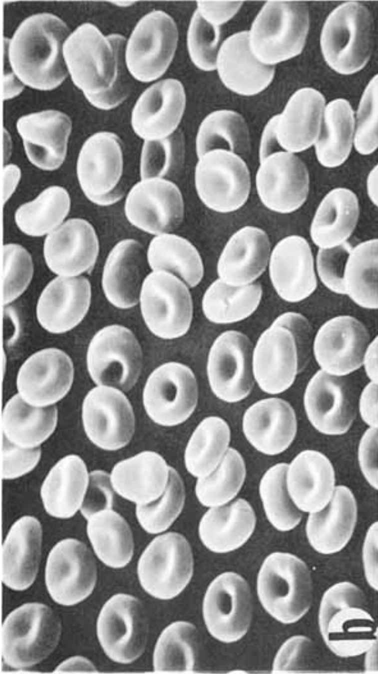
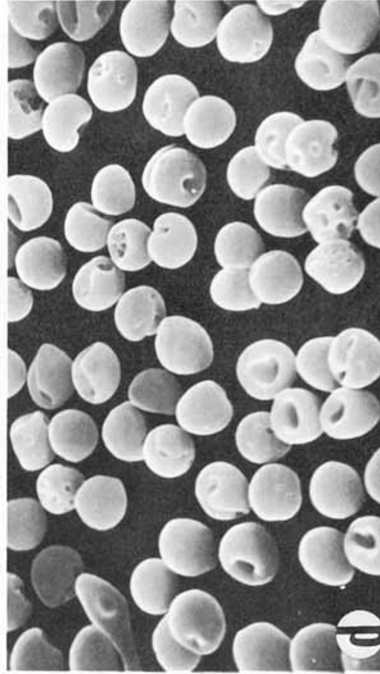
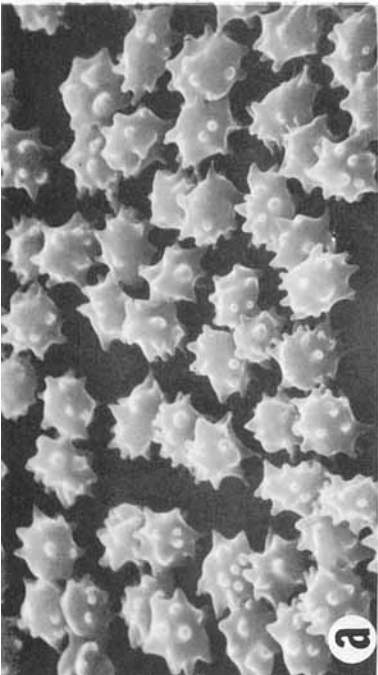
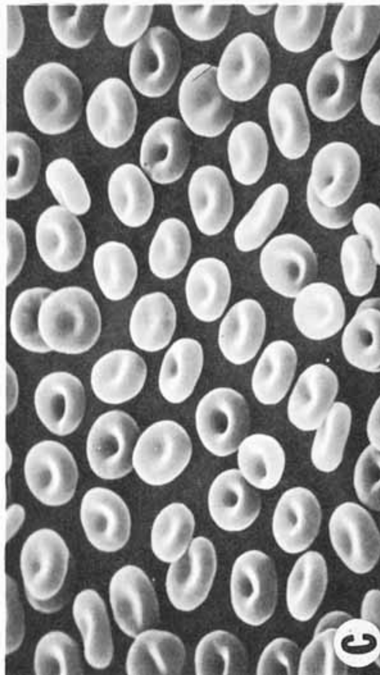


Fig. 1. Radioactive LPC content of red blood cell membranes as a function of incubation time. The total LPC content of the red cells (○) was obtained after four saline washes and the inner leaflet LPC (▲) after four albumin washes. The outer leaflet LPC (●) is calculated by subtracting the residual counts after albumin washes from those after saline washes. Data from two separate experiments using red cells from two different donors are presented and are indicated by the separate characters.

acylation of the incorporated LPC. Since PC does not induce either echinocytic or stomatocytic shape changes and the PC synthesized by acylation accounts for less than 4% of the naturally occurring PC in red cell membrane, its role in red cell shape changes was not considered significant.

Shape Changes

Red cell morphologic changes at various stages of incubation are shown in Figure 2. Five minutes after the start of incubation the red cells were transformed into echinocytes (spherical cells with uniformly distributed spicules [7]); they retained this shape when washed with saline, but resumed their discocytic shape when washed with albumin. In contrast, the morphology of the red cells at 7.5 h was discocytic and remained so after saline washing, but after albumin washing the shape became stomatocytic (stomatocyte III: deeply cup-shaped cells with membrane invagination [7]). Between 5 min and 7.5 h there was a gradual reversal of the morphology of red cells from echinocytic (echinocyte III) to discocytic in spite of the progressive increase in total LPC content. At the same time


b

d

a

c

intervals after albumin washing, the morphology changed from discocytic to stomatocytic, with progressive accumulation of albumin-inaccessible LPC.

In summary, these results demonstrate that five minutes after the start of incubation, the discocytes were transformed into echinocytes with 160 nmoles LPC/ml RBC found in the outer half of the bilayer, and no LPC in the inner half. At 7.5 h 160 nmoles LPC/ml RBC was still present in the outer half as consequence of the equilibrium partition of LPC between the suspending medium and the outer half; however, 20 nmoles LPC/ml RBC was now present in the inner half and the red cells were transformed back into discocytes. This indicates that the echinocytic effect of the LPC in the outer monolayer had been nullified or counterbalanced by the much lower concentration of LPC which had now entered the inner monolayer. Albumin washing of these discocytes produced stomatocytes with 20 nmoles LPC/ml RBC remaining in the inner monolayer and none in the outer monolayer, showing the morphologic effect of unbalanced LPC in the inner leaflet.

DISCUSSION

These results demonstrate the different echinocytogenic (with LPC outside) and stomatocytogenic (with LPC inside) effects that occur when this naturally occurring agent is localized in different areas of the bilayer. They further emphasize the importance of the balance between the inner and outer leaflet concentrations of this agent in determining cell shape. The increasing concentration of LPC in the inner monolayer over the period of observation appears to have been due to its translocation from the outer to the inner leaflet as suggested by previous studies [8].

The kinetics observed for LPC translocation during this study agree well with those obtained for PC translocation using other techniques [10, 11]. The translocation of other echinocytic agents from the outer half of the bilayer to the inner half could also explain the spontaneous reversal of spheroechinocytes into discocytes, as reported by Hoffman [12].

Our observation that shape changes produced by a higher concentration of LPC in the outer monolayer can be balanced by a much lower concentration of LPC in the inner monolayer indicates a differential concentration effect of LPC on cell shape in the inner and outer halves of the bilayer. It has also been reported [9] that a ten-fold increase in cellular concentration of anionic phenothiazines was needed to produce sphero-echinocytes, compared with the concentration of cationic phenothiazines needed to produce spherostomatocytes. Data from both these studies show that an equivalent concentration level of these compounds in the inner and outer monolayers, and the consequent simple equivalent area expansion of each monolayer as previously proposed [5], is insufficient to wholly explain these morphologic changes. The differential concentration

Fig. 2. Red blood cell morphology as a function of incubation time. a) Scanning electron micrograph of echinocytes after 5 min incubation in the medium containing LPC; b) discocytes obtained by washing these echinocytes with 1% albumin solution; c) discocytes which reformed after 7.5 h incubation in the medium containing LPC; d) stomatocytes obtained by washing these discocytes at 7.5 h with 1% albumin solution.

dependence also suggests that either differential charge effects, or surface tension effects of the shape-active agents in the two halves of the bilayer, may mediate these changes.

ACKNOWLEDGMENTS

The work was supported in part by US Public Health Service grants AM 16095 and HL 07100 and by career development award AM 37237 to S.B.S.

REFERENCES

1. Rothman ME, Lenard J: Science 195:743, 1977.
2. Bretscher MS: Nature (New Biol) 236:11, 1972.
3. Gordesky SE, Marinetti GV: Biochem Biophys Res Commun 50:1027, 1973.
4. Verkleij AJ, Zwaal RFA, Roelofson B, Comfurius P, Kastalijn D, Van Deenen LLM: Biochim Biophys Acta 323:178, 1973.
5. Sheetz MP, Singer SJ: Proc Natl Acad Sci USA 71:4457, 1974.
6. Sheetz MP, Singer SJ: J Cell Biol 70:247, 1976.
7. Bessis M: In Bessis M, Weed RI, Leblond PF (eds): "Red Cell Shape." New York: Springer-Verlag, 1973, pp 1–25.
8. Shohet SB: J Clin Invest 49:1668, 1970.
9. Mohandas N, Feo C: Blood Cells 1:375, 1975.
10. Rousselet A, Guthmann C, Matrican J, Bienvenue A, Devaux P: Biochim Biophys Acta 426:357, 1976.
11. Renooij W, Van Golde LMG, Zwaal RFA, Van Deenen LLM: Eur J Biochem 61:53, 1976.
12. Hoffman JF: In Bessis M, Weed RI, Leblond PF (eds): "Red Cell Shape." New York: Springer-Verlag, 1973, pp 51–54.