Abnormalities in the erythrocyte membrane in acute lymphoid leukaemia

Manikuntala KUNDU,* Joyoti BASU,* Parul CHAKRABARTI*† and Madan Mohan RAKSHIT‡

*Department of Chemistry, Bose Institute, 93/1 A.P.C. Road, Calcutta – 700 009, India, and ‡Haematology Unit, N.R.S. Medical College and Hospital, 138 A.J.C. Bose Road, Calcutta – 700 014, India

Erythrocytes from patients suffering from acute lymphoid leukaemia (ALL) show decreased proportions of spectrin tetramers and altered spatial distribution of band 4.1 and ankyrins. These abnormalities of the cytoskeleton are probably responsible for altered membrane fluidity and transbilayer distribution of phosphatidylethanolamine in ALL. ALL is associated with severe anaemia and usually, but not always, with overproduction of lymphocytes. To our knowledge, this is the first report of abnormalities in the erythrocyte membrane in ALL which may, in part, be responsible for the observed anaemia.

INTRODUCTION

The molecular architecture of the erythrocyte membrane displays an asymmetric lipid bilayer, with aminophospholipids mainly confined to the inner leaflet and choline phospholipids to the outer leaflet [1]. Underlying this bilayer, there is a protein network called the membrane skeleton, which is responsible for maintaining the discoid shape and mechanical stability of erythrocytes [2,3]. The skeleton is composed primarily of spectrin tetramers, actin, band 4.1 and some other minor proteins. The lipid bilayer and the cytoskeleton are held together mainly by two proteins, ankyrin and band 4.1 [4,5]. An ATP-dependent protein has been shown to be involved in the selective accumulation of aminophospholipids in the inner leaflet of the bilayer of human erythrocytes [6]. Specific interactions between aminophospholipids and skeletal proteins stabilize the bilayer asymmetry [7–9]. Defects and deficiencies in the skeletal components have been described in certain hereditary haemolytic anaemias, where they give rise to mechanically and/or thermally unstable erythrocytes [10] with shortened life-spans. Important defects in spectrin dimer self-association have been identified in hereditary elliptocytosis [11] and pyropoikilocytosis [12]. Alterations in the association of cytoskeletal proteins and marked structural changes in the membrane lipid asymmetry may lead to important biological and clinical consequences, as evidenced in the case of malaria parasite-mediated infection [13] and sickle-cell anaemia [14]. Acute lymphoid leukaemia (ALL) is a malignant disease of primitive lymphopoietic progenitors. The leukocyte count is usually increased in ALL, but in about one third of patients the total count is normal or low [15]. ALL is associated with severe anaemia. No studies have so far been carried out on the erythrocyte membrane in ALL. We report here some abnormalities in the skeletal protein organization as well as alterations in transbilayer distribution of phospholipids in the erythrocyte membrane, which may, in part, account for the anaemia associated with this disease.

EXPERIMENTAL

Materials

Reagents for gel electrophoresis, dimethyl adipimidate (DMA), trinitrobenzenesulphonic acid (TNBS), diphenylhexatriene and 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Sepharose 4B was obtained from Pharmacia Fine Chemicals, Stockholm, Sweden. All other reagents used were of analytical grade. Blood and bone marrow samples were obtained from four patients in the age group 12–30 years from N.R.S. Medical College and Hospital, Calcutta. Acute leukaemia was diagnosed in all of these patients by standard clinical features. The leukocyte count was 5600–32 600/ μ l. None of the patients had received therapy when blood samples were taken in heparin for this investigation.

Preparation of ghosts and extraction of membrane skeletal components

Erythrocytes were lysed in cold 10 mM-Tris/HCl, pH 7.4. Membranes were sedimented at 22000 *g* for 15 min at 4 °C, and washed three times in the same buffer to remove haemoglobin. Membrane skeletal components were extracted according to Gratzer [16].

Erythrocyte ghosts in 1 mM-Tris/HCl, pH 8.0, containing 0.1 mM-EDTA and 0.1 mM-phenylmethanesulphonyl fluoride were dialysed against the same buffer for 20–24 h at 4 °C. Membranes were sedimented and the supernatants were fractionated on a Sepharose-4B column equilibrated against the same buffer.

Crosslinking with DMA

Ghosts were treated with the bifunctional crosslinking agent DMA as described by Ji & Nicholson [17]. Ghosts in Tris-buffered saline (10 mM-Tris/HCl/0.15 M-NaCl, pH 7.4) were incubated with 0.1 mg of DMA/ml for 60 min at room temperature. The reaction was terminated with 0.05 M-ammonium acetate. The membranes were pelleted at 22000 *g* for 15 min and washed once in

Abbreviations used: ALL, acute lymphoid leukaemia; DMA, dimethyl adipimidate; TNBS, trinitrobenzenesulphonic acid; DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid); *P*, steady-state fluorescence polarization.

† To whom correspondence should be sent.

Fig. 1. Elution pattern of cytoskeletal proteins from normal and ALL erythrocyte ghosts at 4 °C under low-ionic-strength conditions from a Sepharose-4B column.

The continuous line represents the elution from normal erythrocyte ghosts, and the broken line represents the elution from ALL ghosts. Peaks 1, 2 and 3 correspond to the spectrin-actin-band 4.1 complex, tetrameric spectrin and dimeric spectrin respectively. Similar elution patterns have been observed for five normal healthy donors and four ALL patients.

10 mM-Tris/HCl, pH 7.4. The crosslinked ghosts were taken in 1% SDS and run on a SDS/7.5% polyacrylamide gel according to Laemmli [18].

Fluorescence studies with diphenylhexatriene

Ghosts prepared from normal and ALL erythrocytes were mixed with 10 μ M-diphenylhexatriene and incubated at 37 °C for 60 min in the dark. Steady-state fluorescence polarization as function of temperature was determined in a Perkin-Elmer fluorescence spectrophotometer at an excitation wavelength of 360 nm and an emission wavelength of 450 nm. The steady-state fluorescence polarization, P , is obtained by: $P = (I_v - I_h)/(I_v + I_h)$ where I_v and I_h are the intensities of the vertically and horizontally polarized components respectively [19].

Trinitrophenylation of erythrocytes

Erythrocytes were labelled with TNBS as described by Gordesky *et al.* [20]. Cells were suspended in 120 mM-NaHCO₃/1.5 mM-TNBS, pH 8.6, for 1.5 h at 20 °C. Incubations were carried out in both the presence and the absence of an anion-channel-protein inhibitor, DTNB [21,22]. The cells were then washed to remove unreacted TNBS, lysed with 12 mM-NaHCO₃/4 mM-NaCl, and the membrane pellet was extracted with chloroform/methanol/0.1 M-HCl (10:10:9, by vol.). The organic phase

Fig. 2. Densitometric scanning of Coomassie Blue-stained gels of ALL erythrocyte ghosts

Coomassie Blue-stained gels were scanned at 590 nm. (a) Untreated normal ghosts, (b) DMA-treated normal ghosts, (c) untreated ALL ghosts, (d) DMA-treated ALL ghosts. Bands have been numbered according to the nomenclature of Fairbanks *et al.* [35]. Ghosts from all four patients showed similar patterns before and after treatment with DMA.

was dried, and phospholipids were analysed by t.l.c. using chloroform/methanol/acetone/acetic acid/water (10:2:4:2:1, by vol.) as the solvent system.

RESULTS AND DISCUSSION

Extraction and fractionation of skeletal components

Treatment of normal erythrocyte ghosts with low-ionic-strength buffer at 4 °C for a prolonged period (24–48 h) and subsequent fractionation on Sepharose 4B leads to three major peaks attributable to the spectrin–actin–band 4.1 complex, tetrameric spectrin and dimeric spectrin respectively [23]. Supernatants from ALL ghosts treated similarly in low-ionic-strength buffer showed a considerable amount of spectrin dimer formation and reduced tetramer formation, compared to normal ghosts (Fig. 1). A reduced proportion of spectrin tetramers and an increased proportion of dimers has been observed in several cases of hereditary haemolytic anaemia [11,24–27]. This is usually associated with loss of mechanical stability and reduced deformability of erythrocytes [28].

Crosslinking with DMA

The arrangement of skeletal proteins in the erythrocyte ghosts was studied using a bifunctional crosslinking agent, DMA, with a span of 0.86 nm. ALL ghosts showed decreases in the amounts of bands 2.1, 2.2, 2.3 and 4.1, and the formation of high molecular mass proteins which could not penetrate the gel (Fig. 2). At the same concentration of DMA, normal ghosts failed to show any crosslinking of these bands. This suggests a spatial redistribution of the skeletal proteins in ALL erythrocytes so that they become crosslinkable by DMA.

Fluorescence polarization studies

Band 4.1 and ankyrin are mainly responsible for anchoring the cytoskeleton to the lipid bilayer. Protein 4.1 is also known to bind preferentially to the cytoplasmic leaflet phospholipids [29]. In order to investigate the effect of a compromised cytoskeleton on the membrane lipid bilayer, the thermotropic behaviour of normal and ALL ghost lipids was studied. Fluorescence polarization (*P*) measurements with diphenylhexatriene as a function of temperature, suggested increased mobilities of lipids in ALL ghosts compared to normal erythrocyte ghosts (Table 1). Values of *P* were lower in the ALL ghosts compared to the normal ghosts at all temperatures studied. This suggests increased fluidity in the ALL ghost membrane. Malaria parasite invasion, which induces marked alteration in the host cell membrane phospholipids and cytoskeleton proteins, has also been reported to affect membrane fluidity [30].

Trinitrophenylation of erythrocytes

The aminophospholipid distribution across the erythrocyte membrane was studied using TNBS, an amino group labelling reagent, as an external probe. It was found that a considerably greater amount of phosphatidylethanolamine was labelled by TNBS in ALL cells compared to the normal cells (Table 2), suggesting the presence of a greater amount of this phospholipid in the outer leaflet of ALL erythrocytes. This may be due to loss of transmembrane phospholipid asymmetry, as observed in the case of malaria-infected erythrocyte

Table 1. Fluorescence polarization (*P*) of normal and ALL erythrocyte ghosts

Values are means $\pm$ s.d. for four determinations with samples from four different patients.

Temperature (°C)	<i>P</i>	
	Normal	ALL
18	0.30 $\pm$ 0.03	0.23 $\pm$ 0.04
24	0.24 $\pm$ 0.03	0.22 $\pm$ 0.03
31	0.23 $\pm$ 0.01	0.21 $\pm$ 0.04
34	0.22 $\pm$ 0.02	0.20 $\pm$ 0.01
37	0.21 $\pm$ 0.01	0.19 $\pm$ 0.02

Table 2. Labelling of erythrocyte phosphatidylethanolamine (PE) with TNBS in the presence and absence of DTNB

Values are means $\pm$ s.d. for four determinations with samples from four different patients.

Sample	[DTNB]	PE (% labelling)
Normal	–	20.1 $\pm$ 1.2
	5 mM	18.2 $\pm$ 0.8
ALL	–	30.0 $\pm$ 1.9
	5 mM	29.3 $\pm$ 1.6

membranes [13], or increased transbilayer movement of phospholipids in ALL erythrocytes. These changes are probably induced by the structural abnormalities of the cytoskeleton in ALL erythrocytes, because the transbilayer distribution and the slow transbilayer movement of phospholipids are known to be stabilized by the membrane skeleton [9,14,31–33].

Defects in the erythrocyte membrane have been detected in several cases of anaemia [34]. A decreased proportion of spectrin tetramers and altered transbilayer distribution of phospholipids, with the accumulation of more aminophospholipids in the outer leaflet leading to a compromised erythrocyte with increased membrane fluidity, may be among the factors contributing to the severe anaemia associated with ALL. The results of the present study also raise the possibility that a basic abnormality in ALL may reside in multipotent haemopoietic stem cells that differentiate into erythrocytes.

This work was supported by a grant from the Department of Science and Technology, Government of India.

REFERENCES

1. van Deenen, L. L. M. (1981) *FEBS Lett.* **123**, 3–5
2. Goodman, S. R. & Shiffer, K. A. (1983) *Am. J. Physiol.* **244**, C121–C141
3. Bennett, V. (1985) *Annu. Rev. Biochem.* **54**, 273–304
4. Anderson, R. A. & Lovrien, R. E. (1984) *Nature (London)* **307**, 655–658
5. Weaver, D. C., Pasternack, G. R. & Marchesi, V. T. (1984) *J. Biol. Chem.* **259**, 6170–6175
6. Devaux, P. F. (1988) *FEBS Lett.* **234**, 8–12

7. Goodman, S. R. & Branton, D. (1978) *J. Supramol. Struct.* **8**, 455–463
8. Franck, P. F. H., Chiu, D. T.-Y., Op den Kamp, J. A. F., Lubin, B., van Deenen, L. L. M. & Roelofsen, B. (1983) *J. Biol. Chem.* **258**, 8435–8442
9. Lubin, B., Chiu, D., Bastacky, B., Roelofsen, B. & van Deenen, L. L. M. (1981) *J. Clin. Invest.* **67**, 1643–1649
10. Marchesi, V. T. (1985) *Annu. Rev. Cell Biol.* **1**, 531–561
11. Lecomte, M. C., Dherny, D., Solis, C., Ester, A., Feo, C., Gautero, H., Boarnier, O. & Boivin, P. (1985) *Blood* **65**, 1208–1217
12. Lawler, J., Palek, J., Lin, S. C., Prchal, J. & Butler, W. V. (1983) *Blood* **62**, 1182–1186
13. Joshi, P., Dutta, G. P. & Gupta, C. M. (1987) *Biochem. J.* **246**, 103–108
14. Chiu, D., Lubin, B. & Shohet, S. B. (1979) *Br. J. Haematol.* **41**, 233–234
15. Henderson, E. S. (1986) in *Hematology* (Williams, W. J., Beutler, E., Erslev, A. & Lichtman, M. A., eds.), pp. 971–980, McGraw-Hill, New York
16. Gratzer, W. B. (1982) *Methods Enzymol.* **85**, 475–480
17. Ji, T. H. & Nicolson, G. L. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 2212–2216
18. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
19. Shinitzky, M. & Barenholz, Y. (1978) *Biochim. Biophys. Acta* **515**, 367–394
20. Gordesky, S. E., Marinetti, G. V. & Love, R. (1975) *J. Membr. Biol.* **20**, 111–132
21. Reithmeier, R. A. F. (1983) *Biochim. Biophys. Acta* **732**, 122–125
22. Toon, R. M., Dorogi, P. L., Lukacovic, M. L. & Solomon, A. K. (1985) *Biochim. Biophys. Acta* **818**, 158–170
23. Ungewickell, E. & Gratzer, W. B. (1978) *Eur. J. Biochem.* **88**, 379–385
24. Evans, J. P., Baines, A. J., Hann, I. M., Al-Hakim, I., Knowles, S. M. & Hoffbrand, A. V. (1983) *Br. J. Haematol.* **54**, 163–172
25. Coetzer, T. & Zail, S. A. (1982) *Blood* **59**, 900–905
26. Burke, B. E. & Shotton, D. M. (1983) *Br. J. Haematol.* **54**, 173–187
27. Lux, S. E. (1979) *Semin. Hematol.* **16**, 21–51
28. Liu, S. C. & Palek, J. (1980) *Nature (London)* **285**, 586–588
29. Sato, S. B. & Ohnishi, S. (1983) *Eur. J. Biochem.* **130**, 19–25
30. Allred, D. R., Sterling, C. & Morse, P. (1983) *Mol. Biochem. Parasitol.* **7**, 29–39
31. Haest, C. W. M., Plasa, G., Kamp, D. & Deuticke, B. (1978) *Biochim. Biophys. Acta* **509**, 21–32
32. Mohandas, N., Wyatt, J., Mel, S. F., Rossi, E. & Shohet, S. B. (1982) *J. Biol. Chem.* **257**, 6537–6543
33. Lubin, B., Chiu, D., Schwartz, R. S., Cooper, B., John, K., Wolf, L. & Lux, S. E. (1983) *Blood* **62** (Suppl.) 34a
34. Rice-Evans, C. A. & Dunn, M. J. (1982) *Trends Biochem. Sci.* **7**, 282–286
35. Fairbanks, G., Stock, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2606–2617

Received 5 October 1988/28 December 1988; accepted 10 January 1989