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Abnormal erythrocyte membrane phospholipid organisation in chronic myeloid leukaemia

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Abstract. The membrane phospholipid organisation in the red cells of humans suffering from chronic myeloid leukaemia has been analysed using the amino-group labelling reagent trinitrobenzenesulphonic acid and the fluid-sensing fluorophore, Merocyanine 540. Unlike the normal human erythrocytes, trinitrobenzenesulphonic acid in intact chronic myeloid leukaemia erythrocytes modified about 30% phosphatidylserine, under controlled conditions. Also, the chronic myeloid laukaemia red cells, but not the normal cells, were found to bind the fluorescent dye Merocyanine 540. These results demonstrate that loss of the transmembrane phospholipid asymmetry in chronic myeloid leukaemia erythrocytes is accompanied by an enhancement in the outer surface fluidity and, therefore, suggest that the red cells membrane phase-state asymmetry originates probably from the asymmetric arrangements of phospholipids across the membrane bilayer.

Keywords. Phospholipid asymmetry; erythrocytes; phase-state asymmetry; chronic myeloid leukaemia.

Introduction

Chronic myeloid leukaemia (CML) is a clonal disorder common to granulocyte, platelet and erythrocyte precursors (Champlin and Golde, 1985; Gale and Cannani, 1985). Our recent studies have shown that the CML erythrocyte spectrin becomes abnormal due to crosslinking of its two subunits *via* disulphide bonds (Kumar and Gupta, 1983). This abnormality in these cells has been found to be associated with the presence of substantial amounts of Phosphatidylserine (PS) in the outer surface of the membrane bilayer (Kumar and Gupta, 1983). We now report that the amino-group labelling reagent, trinitrobenzenesulphonic acjd (TNBS),- can readily modify about 30% PS in the intact CML erythrocytes. In addition, we show that the fluid-sensing probe, Merocyanine 540 (Mc 540), binds the CML cells but not the normal erythrocytes. These results indicate that the membrane skeletal defects in CML erythrocytes are associated not only with loss of the transmembrane phospholipid asymmetry but also lead to an increase in the outer monolayer fluidity.

Materials and methods

All the chemicals and reagents used in this study were of the highest purity available. TNBS, bovine serum albumin (fatty acid free) and 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) were purchased from Sigma Chemical. Company, St. Louis, Missouri, USA.

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Abbreviations used: CML, Chronic myeloid leukaemia; CML erythrocytes, red cells of humans afflicted with CML; PS, phosphatidylserine; TNBS, trinitrobenzenesulphonic acid; Mc, Merocyanine 540; DTNB, 5,5'-dithiobis (2-nitrobenzoic acid); PE, Phosphatidylethanolamine.

Mc 540 was a gift from Dr. Rolf D. Walter, Bernhard Nocht Institute, Humburg, Federal Republic of Germany. Fetal calf serum was from Difco, USA.

Blood from leukaemic male patients (aged 35-50 years) and from healthy adult donors was obtained from King George's Medical College, Lucknow, and drawn by venepuncture into heparinized glass tubes. All the patients studied here were esta blished cases of CML as shown by their haematological and clinical analysis. Erythrocytes from whole blood were isolated as described earlier (Kumar and Gupta, 1983).

Labelling of erythrocytes with TNBS

Erythrocytes were labelled with TNBS essentially according to the method of Gordeski *et al.* (1975). Incubations were done for varying periods of time (3,6 and 9 h) at 10°, 20° and 30°C. At 20°C, incubations were carried out both in the presence and absence of an anion channel protein inhibitor, DTNB (Reithmeier, 1983; Toon *et al.,* 1985). Cells from incubation mixtures were harvested by centrifugation and washed several times with saline (pH 7·4). Lipids from washed cells were extracted and chromatographed on silica gel G-60 thin-layer chromatography plates according to the published procedures (Kumar and Gupta, 1983) and the percentage of TNBS labelling was determined as described earlier (Gupta and Mishra, 1981).

Binding of erythrocytes with Mc 540

Studies on binding of Mc 540 to both normal human and CML erythrocytes were carried out according to Schlegel *et al.* (1980). The optimum conditions of dye concentrations, duration of incubation and serum concentration were established so that no fluorescence was observed in the normal cells due to non-specific adherence of the dye. Using these conditions, the binding of Mc 540 to CML erythrocytes was carried out and compared. Fluorescence microscopy was performed with a Zeiss microscope using epillumination with green excitation filter BP 546/12 and barrier filter LP 590. Erythrocytes were photographed using ASA 400 Kodak VR film.

Results

Erythrocyte aminophospholipid labelling with TNBS

The amino-group labelling reagent, TNBS, has widely been used as an external membrane probe to determine the transbilayer amino-phospholipid distributions in the membranes of various types of cells (reviewed by Etemadi, 1980; Roelofsen, 1982). However, conditions need to be optimised for using this reagent in the studies of phospholipid distribution across the erythrocyte membrane, as it is known that TNBS penetrates into the human red cells upon prolonged incubations and/or at temperatures > 20°C (Gordesky *et al.,* 1975; Haest *et al.,* 1981). Therefore, normal as well as CML erythrocytes were treated with the reagent at various temperatures for varying periods of time. Table 1 shows that at 10° and 20°C, TNBS modified only PE in the normal cells and both PE and PS in the CML cells; although, labelling at

Table 1. Labelling of erythrocytes with TNBS.

Sample	Incubation temperature (°C)	Incubation time (h)	Labelling of aminophospholipids	
			PE (%)	PS (%)
Normal erythrocytes	10	9	16·4 ± 1·6	0
	20	3	22·4 ± 2·9	0
		6	24·1 ± 3·2	0
		9	25·3 ± 3·1	0
	30	3	24·2 ± 2·6	40·4 ± 2·0
		6	39·2 ± 3·0	45·2 ± 3·9
		9	50·8 ± 1·6	54·6 ± 3·8
CML erythrocytes	10	9	27·9 ± 2·2	24·7 ± 0·8
	20	3	30·1 ± 2·6	28·7 ± 1·8
		6	35·8 ± 2·5	40·8 ± 1·9
		9	42·3 ± 1·2	44·0 ± 0·9

Values are mean of 6-8 determinations ± S.D.

10°C was a trifle inhibited as the amount of phosphatidylethanolamine (PE) labeled in the normal cells was less than that reported to be present in the outer monolayer (Gordesky *et al.,* 1975). However, raising the incubation temperature to 30°C resulted in complete lysis of CML erythrocytes and in labelling of both PE and PS in the normal cells. Moreover, even at 20°C the amounts of labelled aminophospholipids increased significantly with time in case of CML erythrocytes. These results indicate that TNBS readily penetrates into both normal and CML erythrocytes at 30°C and in the CML cells only upon prolonged incubation at 20°C. The labelling at 20°C of CML cells could not be continued to saturation as incubation of these cells beyond 9 h, resulted in heavy lysis.

Penetration of TNBS into red cells is known to be inhibited upon blocking the red cells anion transfer system by an appropriate anion channel protein inhibitor (Haest *et al.,* 1981). Therefore, we treated the red cells with TNBS at 20°C in the presence of an anion channel protein inhibitor, DTNB (Reithmeier, 1983; Toon *et al.,* 1985). Table 2 shows that under these conditions about 19% PE was modified in the normal cells whereas in CML erythrocytes, about 26% PE and 30% PS were labelled. These amounts of labelled amino-phospholipids are comparable to those

Table 2. Labelling of erythrocytes with TNBS in the presence of DTNB

Sample	DTNB (mM)	Labelling of aminophospholipids	
		PE (%)	PS (%)
Normal erythrocytes	5	18·8 ± 0·3	0
	10	19·1 ± 0·6	0
CML erythrocytes	5	26·3 ± 0·5	29·1 ± 0·2
	10	25·6 ± 2·0	33·6 ± 3·4

Incubation were done at 20oC for 9h.
Values are mean of 4 determinations ± S.D.

labelled by TNBS at 10°C or 20°C (3 h) in the absence of DTNB. From these findings, it may be concluded that the typical transmembrane phospholipids asymmetry in erythrocytes (Schwartz *et al.,* 1985) is lost during CML. This is quite consistent with the earlier studies (Kumar and Gupta, 1983).

Mc 540 binding to erythrocytes

Mc 540 is a negatively charged fluorescent dye, having the unique characteristic feature to show enhanced fluorescence on intercalation with the hydrophobic dom ain of the bilayer, and it binds preferentially to relatively disordered or fluid domains in the outer leaflet of the membrane bilayer in intact cells (Williamson *et al.,* 1982). The dye has a low affinity for the normal red cell membrane, and even this affinity is abolished by the addition of a competing serum at a concentration of 5%. For this purpose, autologus plasma, AB serum, fetal calf serum or even bovine serum albumin can be used. Of the various sera tested, AB serum was found to give the best results. The optimum results were obtained by using 5% AB serum and a dye concentration of 20 μg/ml cell suspension. Incubation of 10^5–10^6 red cells/ml for 10 min at 37°C was adequate. Under these conditions, 40-60% CML erythrocytes were stained with the dye whereas the normal red cells completely failed to fluoresce after the Mc 540 treatment (figure 1). These results strongly indicate that fluidity of the outer leaflet of the erythrocyte membrane bilayer is enhanced in CML.

Discussion

Erythrocyte membrane phospholipids are asymmetrically distributed across the membrane bilayer (reviewed by Schwartz *et al.,* 1985). Phosphatidylcholine and sphingomyelin are localized mainly in the outer monolayer whereas PE and PS are present almost exclusively in the inner monolayer. This typical transmembrane phospholipid asymmetry was absent in CML erythrocytes, since considerably larger amounts of aminophospholipids were found to be located in the external monolayer of these cells, as compared with the normal human erythrocytes. In the normal cells, about 19% PE and 0% PS were labelled by TNBS whereas in the CML cells, this reagent modified about 26% PE and 30% PS. These amounts of the labelled aminophospholipids should represent the external phospholipids, as similar amounts of these lipids have earlier been shown to be accessible to phospholipase A_2 in the intact CML erythrocytes (Kumar and Gupta, 1983).

Human red cell membrane choline-phospholipids are known to be more saturated than the aminophospholipids (Williams *et al.,* 1966). Therefore, movements of PE and PS from the inner to the outer monolayer should lead to an increase in the outer surface fluidity. This is quite consistent with the present observation that Mc 540 readily binds to the CML erythrocytes but fails to stain the normal red cells.

This study demonstrates that during CML loss of the transmembrane phospho lipid asymmetry in erythrocytes is associated with an increase in the outer monolayer fluidity. These membrane changes in the cells are probably induced by the structural defects in the membrane skeletal proteins (Kumar and Gupta, 1983), as associations of these proteins with the membrane bilayer seem to stabilize the membrane phos pholipid asymmetry in the red cells (Haest, 1982). Also, our results corroborate the

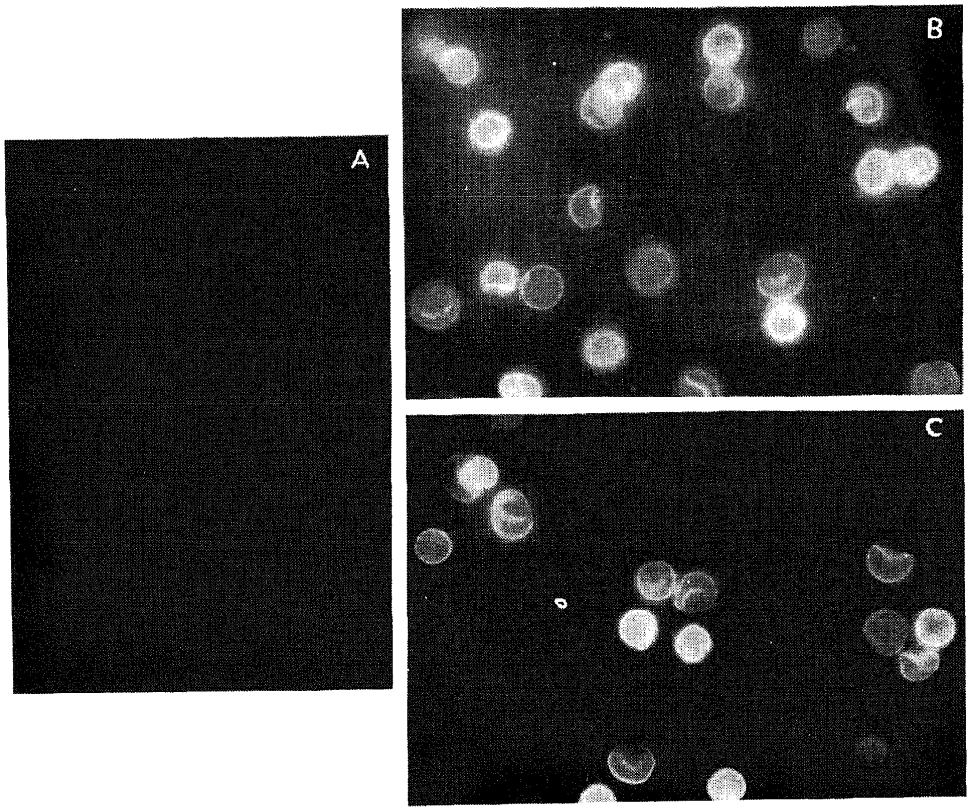

Figure 1. Staining of human erythrocytes with Mc 540. All the cells were photographed at the same magnification and exposure time of 30 s. A, Normal erythrocytes; B and C, CM erythrocytes. Note that the membranes of CML erythrocytes fluoresce after Mc 540 staining but under identical conditions, the normal red cells do not show any fluorescence.

earlier studies which showed that differential fluidities across the erythrocyte membrane are due to asymmetric transbilayer distributions of the membrane phospholipids (Williamson *et al.,* 1982).

Further, it has earlier been suggested that alterations in the membrane phospho lipid asymmetry leads to several pathological disorders (Schwartz *et al.,* 1985). Severe anaemia in CML patients could be due to the externalisation of PS, as it would accelerate the distruction of the CML red cells by the macrophages (Schroit *et al.,* 1985).

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