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Abnormalities in erythrocyte membrane band 3 in chronic myelogenous leukemia

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The anion transport activities of erythrocytes from patients with chronic myelogenous leukemia (CML) and normal donors were comparable. In CML erythrocytes, significant reduction in the number of ankyrin-binding sites, present in the cytoplasmic domain of band 3, may lead to partial loss of cytoskeletal anchorage to the bilayer and account for their increased Con-A agglutinability and heat-sensitivity (Basu, J., Kundu, M., Rakshit, M.M. and Chakrabarti, P. (1988) Biochim. Biophys. Acta 945, 121–126).

Band 3 is the major integral membrane protein of mammalian erythrocytes [1,2]. The band 3 gene has been sequenced from human [3], murine [4] and chicken [5] erythrocytes. This 95 kDa polypeptide possesses two distinct structural domains. The transmembrane domain with the carboxy-terminus bears the anion transport function [6,7] and the amino-terminal cytoplasmic domain serves as an anchor for the membrane cytoskeleton and the glycolytic enzymes [8,9]. The cytoskeleton is responsible for maintaining the biconcave shape of the erythrocyte, its deformability and stability [10–12]. Spectrin tetramers interact with actin, protein 4.1 and adducin to form the cytoskeleton network [13–15] which is linked to the membrane by interactions of protein 4.1 with glycophorin [16] and ankyrin with band 3 [17].

Chronic myelogenous leukemia (CML) is a hematologic malignancy characterized by excessive growth of myeloid cells and their progenitors [18]. Our previous studies have shown that CML erythrocytes contain a reduced proportion of spectrin tetramers, show increased thermal sensitivity, significant organizational modification of cytoskeletal components and enhanced concanavalin-A-induced agglutinability [19]. The last observation suggests some alteration in the anion-transport protein, which is the concanavalin A receptor.

While there are several reports on the abnormalities of cytoskeletal proteins in pathological erythrocytes, this is, to our knowledge, the first report of alterations in a functionally important domain of band 3 associated with chronic myelogenous leukemia.

Blood from leukemic patients was obtained in heparin from NRS Medical College and Hospital, Calcutta. Blood was also collected from normal, healthy volunteers. For sulfate efflux studies, cells were washed twice in Hepes buffer (10 mm Hepes/75 mM KCl/50 mM Na_2SO_4/40 mM sucrose (pH 7.4)).

The $^{35}SO_4^{2-}$ self-exchange rate was determined following the method of Cabantchick and Rothstein [1]. Erythrocytes were washed in Hepes buffer and then loaded with $^{35}SO_4^{2-}$ by incubation for 2 h at 37°C in Hepes buffer containing trace amounts of $Na_2^{35}SO_4$ at 25% hematocrit. The washed $^{35}SO_4^{2-}$-loaded cells were suspended in Hepes buffer at 5% hematocrit, and P_t, the radioactivity at different times, was determined by removing 1 ml of cell suspension at 5, 10, 20, 30 and 45 min, centrifuging each sample and counting 0.2 ml of the supernatant. P_∞, the radioactivity at infinite time was determined by adding 0.2 ml of trichloroacetic acid (30% w/v) to 1 ml of the suspension and counting the radioactivity of the supernatant after centrifugation. Rate constants were obtained from the slopes of the lines obtained by plotting $(P_\alpha - P_t)/P_\alpha$ vs. time.

Inhibition studies with DIDS were carried out by incubating $^{35}SO_4^{2-}$-loaded cells with different concentrations of DIDS for 30 min at 37°C. Unreacted DIDS was removed by washing the cells three times with Hepes buffer [20]. Sulfate efflux was determined as described above.

Abbreviations: CML, chronic myelogenous leukemia; DIDS, 4,4′-diisothiocyanostilbene 2,2′-disulfonate.

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For the study of ankyrin binding to ankyrin depleted vesicles, ankyrin was purified from human erythrocyte ghosts from normal volunteers by the method of Bennett [21]. Proteinase inhibitors were added at each step of purification, and the final ankyrin preparation was found to be homogeneous on SDS-PAGE. This preparation was labeled with ^{125}I-labeled Bolton-Hunter reagent as described by Bennett [21].

Ankyrin-depleted vesicles were prepared from normal and CML erythrocytes according to Bennett and Stenbuck [8]. Briefly, ghosts were extracted successively with a minimum of 10 vol. each of 0.2 mM EDTA (pH 7.5) and then 1 M KI/7.5 mM sodium phosphate/1 mM sodium EDTA/1 mM dithiothreitol (pH 7.5) adding 20/μg phenyl-methylsulfonyl fluoride per ml at each step. The resulting ankyrin-depleted vesicles were collected by centrifugation for 40 min at 19000 rpm in an SS-34 rotor.

Binding of ^{125}I-ankyrin to KI-extracted vesicles was studied according to Bennett [21]. ^{125}I-ankyrin (5–100 μg/ml, > 100000 cpm/μg) was incubated for 90 min at 25°C with 1–40 μg membrane protein in a volume of 0.22 ml 0.1 M KCl/7.5 mM sodium phosphate/0.2 mM sodium EDTA/0.2 mM dithiothreitol/10% sucrose (w/v) (pH 7.5). Free and membrane-bound ankyrin were separated by layering 0.2 ml of the sample over 20% sucrose in assay buffer in Eppendorf microfuge tubes followed by centrifugation at 18000 rpm for 30 min in an SS-34 rotor. Radioactivity was determined in the membrane pellets. Binding of heat-denatured (10 min, 70°C) ankyrin was measured and subtracted in each case to account for nonspecific binding.

In the light of their enhanced Con A agglutinability as shown in our previous study [19], it was important to assess whether there were any alterations in band 3 of functional significance in CML erythrocytes. The anion-transporting function of band 3 is vested in its transmembrane domain. Hence the SO_4^{2-} self-exchange efflux rates were measured in normal and CML erythrocytes in order to compare their anion-transport capabilities.

Comparable rate constants of $^{35}SO_4^{2-}$ efflux in normal and CML erythrocytes (Fig. 1) suggest no significant alterations in the anion transport site of band 3. Stilbene disulfonate derivatives such as DIDS are potent inhibitors of anion transport and bind to either one or two lysine residues accessible only from the extracellular side of the membrane [22–24], neither of which participates directly in anion transport. The comparable rate constants in normal and CML erythrocytes in the presence of various concentrations of DIDS (Table I) suggest that there is probably no significant structural or conformational alteration of band 3 near the DIDS-binding domain.

Since ankyrin is the key protein involved in the interaction of cytoskeletal network to band 3, binding

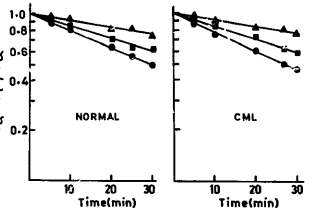

Fig. 1. Effect of DIDS treatment on the $^{35}SO_4^{2-}$ self-exchange rate in normal and CML erythrocytes ●, no DIDS; ■, 2 μM DIDS; ▲, 5 μM DIDS. Inhibition by DIDS was studied as described in the text. Data represent the mean of determinations with four normal and four CML individuals.

of ^{125}I-labeled ankyrin, isolated from normal erythrocytes, was studied with the ankyrin-depleted membrane vesicles prepared from both the normal and CML erythrocytes. Scatchard analysis (Fig. 2) for the study of ankyrin binding was biphasic with a high- as well as a low-affinity component. The data showed significantly reduced ^{125}I-ankyrin binding for all points in the high-affinity region of each profile. Estimates of high-affinity ankyrin binding sites in normal membranes ranged from 39 to 45 μg/mg membrane protein, whereas for the CML patients estimates were consistently lower, ranging from 9 to 15 μg/mg membrane protein (Table II). Points in the low-affinity region of the Scatchard plots approach the abscissa asymptotically and were prone to error.

The significant reduction in the number of ankyrin binding sites in CML erythrocytes reveals a marked alteration in the cytoplasmic domain of band 3. Since the binding of ankyrin to band 3 is primarily responsible for anchoring the cytoskeleton to the bilayer, it is tempting to speculate that the increased thermal sensitivity of CML erythrocytes [19] may be due to partial loss of anchorage of the cytoskeleton to the membrane.

TABLE I

Effect of DIDS treatment on the $^{35}SO_4^{2-}$ self-exchange efflux rate constant (k_t) in normal and CML erythrocytes

Values represent the mean of four determinations with four normal or four CML individuals ± S.D. * In parentheses: percent inhibition = 100 (k_t of control cells − k_t of DIDS-treated cells)/k_t of control cells.

Cell type	$-k_t$ (min^{-1}) at [DIDS] (μM) of:		
	0	2	5
Normal	0.017 ± 0.0001	0.013 ± 0.0008 (23.50)	0.008 ± 0.0004 (52.90)
CML	0.018 ± 0.004	0.013 ± 0.0002 (27.70)	0.007 ± 0.0003 (61.10)

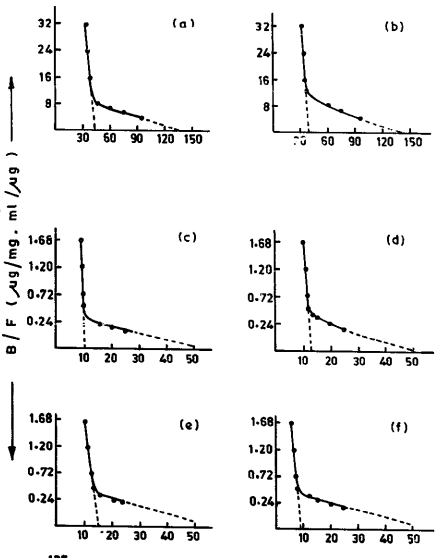

Fig. 2. Scatchard analysis of ^{125}I-ankyrin binding to KI-extracted vesicles from two normal (a and b) and four CML (c, d, e and f) individuals. Binding studies were carried out as described in the text. B, bound ankyrin (μg/mg membrane protein); F, free ankyrin (μg/ml).

TABLE II

Ankyrin binding by KI-extracted vesicles from normal volunteers and CML patients

Results represent the mean ± S.D. of three separate determinations.

Individual	Number of high-affinity ankyrin-binding sites (μg/mg membrane protein)
Normal	
1	45 ± 2
2	39 ± 3
3	41 ± 3
CML	
1	10 ± 2
2	13 ± 1
3	15 ± 4
4	9 ± 2

The control of protein mobility in membranes may play a key role in regulation of functional activities of membrane receptors. It has been suggested that band 3 molecules that are not bound to the cytoskeleton are rotationally and translationally mobile, and the long-range translational mobility of band 3 is restricted by spectrin tetramers [25]. Since the number of ankyrin-binding sites (Table II) and the proportion of spectrin tetramers [19] is reduced in CML erythrocytes, both these factors may lead to an increased lateral mobility of band 3 in this case, and account for enhanced Con A agglutinability. The rearrangement of band 3 into clusters provides the recognition site for antibodies against senescent cells. These antibodies bind to remove aged cells from the circulation [26,27]. It may therefore be speculated that clustering of band 3 in CML erythro-

cytes leads to their removal from circulation, accounting in part for the anemia associated with the disease. The role played by the cytoplasmic domain of band 3 in interacting with cytoskeletal components, might be altered due to one or a combination of reasons, including changes in its primary sequence, conformational changes or control mechanisms such as phosphorylation, etc. This remains to be investigated.

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