Upsala J Med Sci 96: 23–33, 1991

Reduction in Band 3 Protein of Red Cells in Sickle Cell Anaemia

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ABSTRACT

Twelve patients suffering from sickle cell anaemia (Hb <75 g/l) with SS-haemoglobin were studied with special regard to Band3-protein content in their erythrocyte membranes on SDS-PAGE. They were compared in this respect with 7 asymptomatic individuals with AS-haemoglobin and 10 healthy controls with the ordinary adult haemoglobin. A heterogeneity in Band 3-protein content was evident among the 12 patients. Six of them belonged to a low content group while the other 6 belonged to a high content group comparable with the asymptomatic subjects and the controls. No simple relationship seemed to exist between Band 3-protein reduction and the clinical presentation.

INTRODUCTION

Although the abnormal composition and behaviour of the sickle cell haemoglobin are the primary elements in sickle cell disease (1) investigators have found several erythrocyte membrane aberrations which are related to the clinical course of the disease. Sickle erythrocyte membranes have been subject to oxidative damage by excessive radical formation (6) and by a reduction in the antioxidant potential of these cells (14). Platt and coworkers (12) reported decreased binding of spectrin to spectrin-depleted inside-out vesicles of sickle cells which was

not explained by quantitative reduction of binding sites. Other proteins reported to be affected were ankyrin and Band 4.1 (13,16). Fung et al (4) speculated that oxidation of Band 3-protein caused an alteration in water exchange in sickle cells. Denatured haemoglobin S was reported to copolymerise with Band 3-protein, glycophorin and ankyrin forming insoluble hemichromes (8,17). These clusters were shown to be a site for autologous IgG binding and possibly a reason for identification and removal of sickle cells (8).

We report here a reduction in Band3-protein in erythrocyte membranes of a subgroup of patients with sickle cell disease.

MATERIAL AND METHODS

12 patients with sickle cell anaemia, 7 subjects with haemoglobin AS (asymptomatic individuals with sickle cell trait) from Sudan and 10 normal Swedish donors (controls) were examined. Blood samples were collected from them in sodium heparin vacutainer tubes (Becton Dickinson, England) after consent. The blood counts were done following standard manual methods and expressed according to SI rules (2). Diagnosis was made by sickling test and haemoglobin electrophoresis on cellulose acetate strips with TRIS-EDTA-borate buffer pH 8.9 (2). The blood was centrifuged and red cells were washed 3 times in isotonic phosphate buffer pH 7.4. The buffy coat was removed by sucking after each centrifugation. The washed cells were frozen in liquid nitrogen and then transported in dry ice by air to Sweden and stored in $-70^{0}C$ freezer for at most 6 months until use.

Membrane preparation. All chemicals used were of the analytical grade. A method described by Dodge et al (3) was followed in preparing the ghosts with a modification as described below. The frozen cells were thawn in cold ($4^{o}C$) isotonic phosphate buffer pH 7.4 (1:1) and immediately taken in the haemolysing hypotonic

phosphate buffer which contained 0.02% (v/v) Triton x-100. Including this low concentration of Triton x-100 was found necessary for the preparation of the membranes from frozen cells to obtain a membrane fraction of the same type as that prepared from fresh red blood cells as shown by the subsequent electrophoretic separation of the membrane proteins. The preparation was centrifuged at 2^{o}C using Beckman ultracentrifuge at 20,000xg for 40 min. The supernatant was sucked and the pelleted membrane material was washed 3 times with the same hypotonic buffer under the same centrifugal conditions. The protein content of the membrane material was measured according to Salo (15). The pelleted membrane material was suspended in a small amount of the hypotonic phosphate buffer after the final wash and frozen at -70^{o}C until use.

Sodium dodecyl sulphate(SDS) polyacrylamide gel electrophoresis (PAGE) (13). The membrane suspension was thawn quickly in a water bath and solubilized in a Tris-acetic acid buffer (0.2M, pH 9.5) containing 2% SDS, 2mM EDTA, 1% dithiothreitol (DTT) and 0.005% bromophenol blue as a tracing dye. A precisely defined amount of soy bean trypsin inhibitor (STI mw 22000 Daltons, Sigma company, St. Louis USA) was always included in the preparation and used as an internal standard for quantitative evaluation of the protein bands in the electropherograms. The samples were heated in boiling water for 5 min, cooled to 22^{o}C and then iodoacetamide (15% w/v) was added followed by incubation for 60 min at 22^{o}C. The samples were either run immediately or kept frozen at -20^{o}C until electrophoresis was done. The Pharmacia PhastSystem (Uppsala, Sweden) was used for the electrophoretic separation. The membrane preparation supplied with the internal standard was applied on the gradient gel, 8-25%. Pharmacia low molecular weight kit was run with some of the samples. Electrophoresis proceeded for a total of 64 aVH (accumulated volt hours). The

gels were stained with Commassie brilliant blue according to Heukeshoven (5) and dried in room temperature. They were subsequently scanned using LKB laser ultroscanner model GXL (LKB Bromma,Sweden). Integration and display were done using LKB software GSXL/1D on an IBM computer.

Calculations. The different peak areas were related to the known internal standard with the formula a+b/b where a is the respective protein band peak area and b is the internal standard band peak area well separated from the rest of the erythrocyte membrane protein bands. This ratio equals the expression "standardized peak area value".

Statistics. Student's t-test was used for calculation of significance. A p-value <0.05 was regarded significant.

RESULTS

Table 1 shows age, and the haematological values of the patients and the AS-asymptomatic individuals from Sudan. The mean age of the patients was distinctly lower (7.7 year) than that of the AS asymptomatic subjects (24.6 year). Also, the mean haemoglobin concentration of the patients was only about half that of AS individuals while the reticulocyte count was remarkably increased (table 1). The controls (Swedish blood donors) were normal with regard to haemoglobin concentration and reticulocyte counts (data not given in table). Figure 1 illustrates SDS-PAGE analysis of erythrocyte membrane proteins from AS individuals (lanes 1-3) and SS patients (lanes 4-6). A reduction of Band 3-protein from SS patients was observed. This finding was confirmed by a laser densitometric scanning of the electropherograms from one the AS individuals (fig 2A) and one of the SS patients (fig 2B). Although the scans of the spectrin heterodimers were about the same a clear reduction of Band 3-protein content of the SS patient was seen.

Table 1. Mean values of age, haemoglobin concentration, packed cell volume (PCV) and reticulcyte counts (retics) of 12 patients (4 males & 8 females) and 7 asymptomatic subjects (AS,3 males & 4 females). Ranges are given in brackets.

	age (years)	Hb (g/l)	PCV (%)	retics (%)
patients (SS)	7.7 (0.3-16.5)	63 (53-75)	21.6 (19-25.5)	14.4 (4-25)
AS subjects	24.6 (5-51)	123 (97-141)	37.4 (30-45)	1.5 (1-2)

The low Band 3-protein content was not a consistent finding among the SS patients; instead, a heterogeneity with regard to standardized peak area value seemed to exist. This was demonstrated by selecting the lowest standardized peak area value of the controls and the asymptomatic AS individuals as a cut-off value. Six patients fell beneath (group A patients) and the other 6 patients (group B patients) fell above the cut-off value (Table 2). Group A patients differed significantly from the controls and the asymptomatic subjects with regard to the standardized peak area value while group B did not. There seemed to be no simple relationship between the amount of Band 3-protein in the erythrocyte membranes of the patients and the clinical features of the disease. However there were two sisters among the patients (data not shown), one falling in group A and the other in group B. The former was older (12 years) and showed very severe form of the disease while the latter was younger (6 years) and had fewer complaints and had never needed blood transfusion. Similarly, there were two infants , 4 and 7 months respectively, with no

overt symptoms who fell in group B.

Table 2. Band 3-protein standardized peak area value (mean ± SD) of patients (all, group A and group B), asymptomatic subjects (AS) and controls (AA).

	Patients			AS subjects	AA contr
	all	group A	group B		
	n=12	n=6	n=6	n=7	n=10
Band 3	1.892	1.672	2.244	2.492	2.180
	0.467	0.402	0.179	0.292	0.169

p<.01 NS NS

p<.01

p<.001

NS

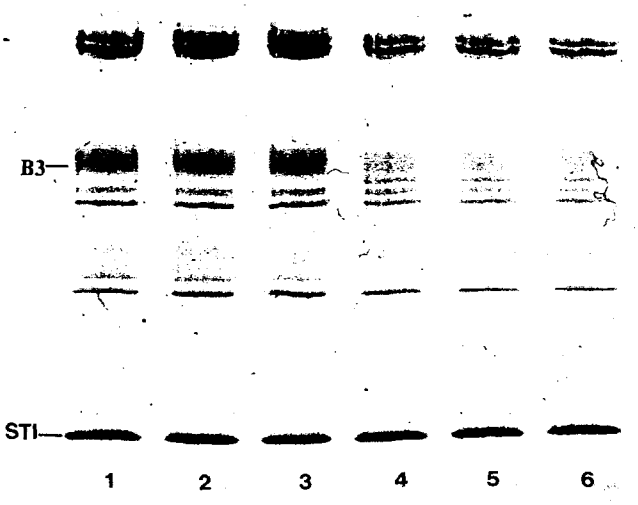


Fig. 1 SDS-PAGE of erythrocyte membrane proteins. Lanes 1-3 AS subject, lanes 4-6 a patient with SS haemoglobin. B3 denotes Band 3 and STI soybean trypsin inhibitor internal standard (mw 20,000 Daltons).

Fig. 2 Laser scan of SDS-PAGE gels from an AS subject (Fig 2A) and an SS patient (Fig 2B). The reduction in the band area corresponding to Band 3 in Fig 2B is evident. STI= soybean trypsin inhibitor (internal standard).

Fig. **2A**

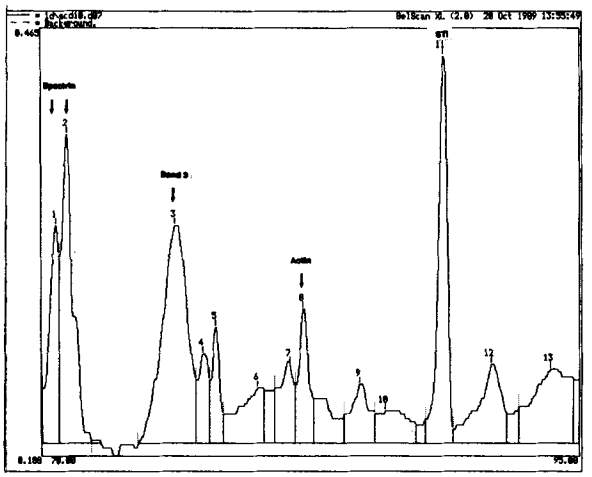
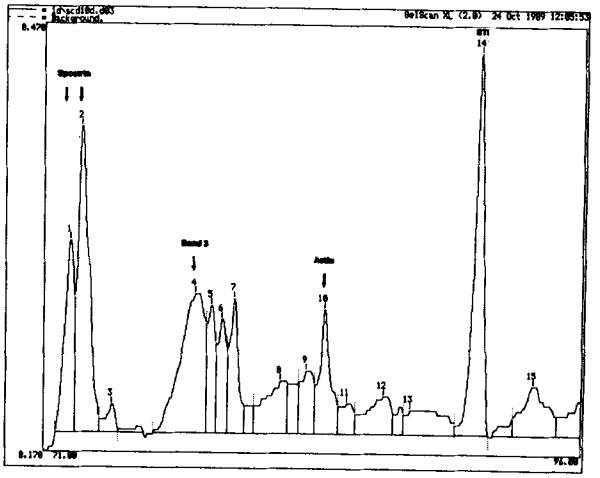


Fig. **2B**



DISCUSSION

This study aimed at finding any possible coupling between the content of the main integral protein (Band 3) in the erythrocyte membranes and the sickle cell disease. The exact amount of the internal standard was always included in the sample to improve the reproducibility of the method.

All our patients presented with the severe type of anaemia with mean haemoglobin levels less than 70 g/l. Although the controls were not age-matched and of different ethnic group, the subjects with haemoglobin AS (asymptomatic individuals) were siblings and parents of the patients. The asymptomatic AS subjects showed no significant difference in results regarding Band 3-protein content compared to the controls. The selection of the cut-off value for Band 3-protein content as the lowest standardized peak area value of the controls and the AS group made it possible to identify 2 distinct groups of the patients, group A being with significantly lower standardized peak area value than group B, controls and the AS subjects. This adds to the heterogeneous behavior of the erythrocytes from patients with sickle cell anaemia. We could not clearly discern any difference between groups A and B with regard to haemoglobin concentration and reticulocyte counts. Still, a correlation may exist between symptoms and membrane findings since the two infants and the sibling with the milder symptoms fell in group B. The reduction in haemoglobin concentration among the SS patients was combined with an increase in reticulocyte counts indicating ongoing haemolysis. However, we did not find any inverse correlation between Band 3-protein content and reticulocyte counts. Kay et al (7) reported reduction in Band 3 protein associated with increased cell aging and haemolytic anaemia. Since Band 3 is considered to have a central role in erythrocyte membrane skeleton stability (9,18) a relationship between Band 3-protein

content and haemolytic tendency would be expected. Platt (11) reported observations on Band 3-protein reduction in sickle cells and postulated that it was due to a release into plasma of spectrin-free vesicles which contained Band 3-protein.

Therefore, it cannot be ruled out that the reduction in Band 3 protein which we found in a subgroup of patients with sickle cell disease may be related to the degree of haemolysis and so the degree of anaemia. A larger controlled study is needed to clarify this possible relationship.

ACKNOWLEDGEMENTS

This work has been supported by the University of Khartoum (Sudan) and the University of Uppsala by a fund from BITS (Sweden). Our thanks to professor Riad Bayoumi and professor Mohamed I.A. Omar from University of Khartoum for supervising this work in Sudan. We are gratefull to Dr. Ingmar Olsson (Pharmacia Uppsala) for kind cooperation with regard to the separation technology. Our thanks are also due to professor C-H de Verdier (Dept. of Clinical Chemistry) and professor Yngve Hofvander (International Child Health Uppsala) for valuable discussions.

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