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Sickle Cell Disease in the Sudan

Clinical and Biochemical Aspects

Minireview Based on a Doctoral Thesis

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INTRODUCTION

Sickle cell anaemia is a haemoglobinopathy due to a single point mutation in the ß-chain of human haemoglobin. The amino acid valine replaces glutamic acid in the sixth position of the ß-globulin chain (71). The homozygous inheritance of this abnormality produces haemoglobin SS and individuals with this genotype suffer from sickle cell anaemia. Subjects with the heterozygous form are designated AS and are essentially healthy unless exposed to extreme conditions (113).

Haemoglobin and sickle cell formation

The normal adult haemoglobin molecule is a tetramer composed of 2 α-chains and 2 ß-chains. Each chain is provided with a haeme molecule which reversibly binds oxygen. The oxygenated molecule is called oxyhaemoglobin and the deoxygenated one, deoxyhaemoglobin. The normal haemoglobin, designated AA, is soluble in both its oxy and deoxy forms. The sickle haemoglobin is less soluble in the deoxygenated form, leading to the characteristic polymer formation which produces the sickle cell. The polymer formation of sickle cell haemoglobin goes through stages of nucleation, growth and gelation, which is a reversible process upon reoxygenation (30). There is a delay in the gelation which enables most cells to pass the venous side for reoxygenation in the lungs without being sickled (79). However, there are 10-30% of the cells which will sickle during each round in the circulation, being less than the expected number by in vitro studies (50,112,79). The polymer formation is the property of haemoglobin S only, and the presence of haemoglobin F especially, as well as the normal adult haemoglobin A, will impede this process (30,84). The polymer deforms the erythrocytes to attain the sickled form which, like the gelation, is a

reversible phenomenon upon reoxygenation. However, some cells, after several cycles of sickling and unsickling, lose the capacity to recover the biconcave shape even in high oxygen tension, probably due to a permanent membrane injury (Figure 1). These cells are called irreversibly sickled cells (ISCs) and their numbers vary from one patient to another, ranging from 2% to 30%. ISCs have been reported to correlate well with the severity of the disease (85).

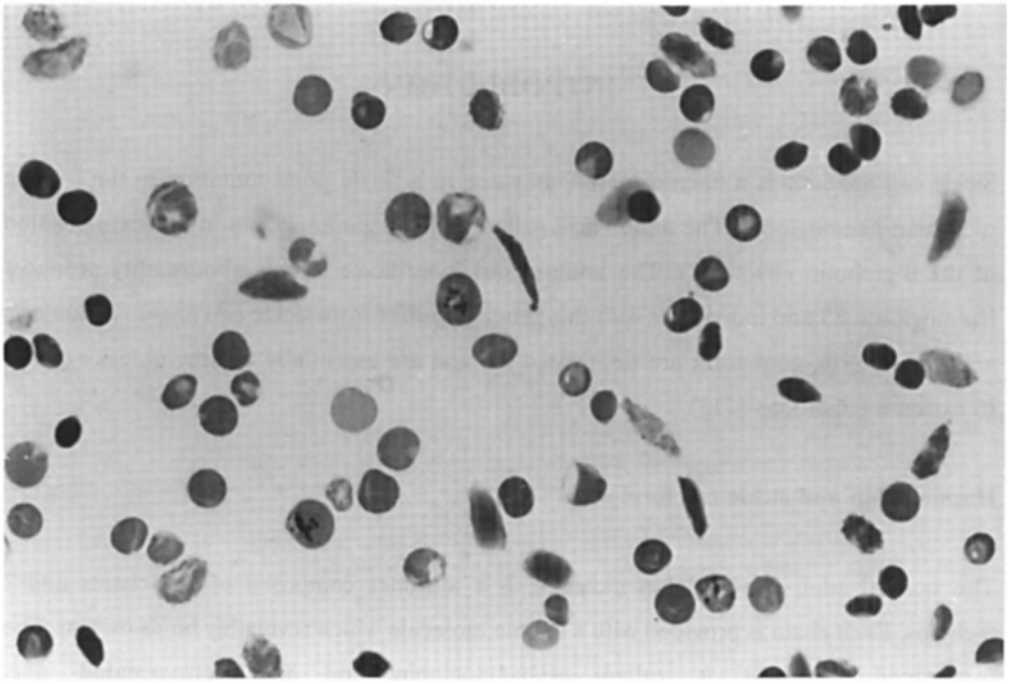


Figure 1. Irreversibly sickled cells in the blood smear of an SS patient, stained for reticulocyte count, x250.

Prevalence and geographical distribution

Sickle cell disease was first described by Herrick in the United States in 1910, when he described "peculiar elongated and sickle-shaped red blood corpuscles in a case of severe anemia" (46). Since then it has been widely described. The sickle cell gene is particularly prevalent in west and central Africa, the Arabian peninsula, parts of India and also in

North and South America and the Mediterranean region, in Greece and Turkey (7,68,113). Now the gene is prevalent anywhere man can live (68).

In North America, about 8% of the Afro-Americans carry the gene (69,110) and an estimated 50000 people have the disease sickle cell anaemia (26,69). The prevalence in Africa has been 25-29% in Nigeria (2,78), 14% in Zaire (81) and 23.8% in lake Victoria region in Kenya (86).

In Sudan, sickle cell anaemia was first reported in 1926 by Archibald (139). Three foci of the disease have been described: Western Sudan, with a prevalence rate of up to 30% among the Baggara tribes (61,125); southern Sudan, where a prevalence rate of up to 18% was found among the southern nilotes (34); and in the Blue Nile Province, central Sudan, a prevalence ranging from 0-5% among the indigenous population and up to 16% was found among the immigrant tribes in the area (3).

The clinical features of the disease

The term sickle cell disease includes a range of abnormal haemoglobins sharing the sickling phenomenon. These include the homozygous sickle cell anaemia (haemoglobin SS), the heterozygous form (AS) and heterozygous inheritance of the sickle cell haemoglobin with another heterozygous haemoglobinopathy like S-ß thalassaemia, SC disease and SO Arab disease.

The clinical picture shows great heterogeneity even in the homozygous SS form. Generally, severe illness is seen in Africa while a mild form compatible with normal life is seen among the Shitti Arabs of eastern Saudi Arabia. Even in the same place, patients with an identical molecular defect display greater variation than expected in the clinical course of the disease (68,119).

Homozygous sickle cell anaemia usually presents with pallor, dactylitis, which is painful and nonpitting swelling of the hands and/or feet (hand-foot syndrome), jaundice and painful crises, mostly in the long bones and abdomen. In Jamaica, a prospective study of 314 patients followed from birth showed that dactylitis was the most common initial symptom found in 40% of all patients (10), followed by painful crises in more than one fourth of the study group. Trowell and coworkers (123) reviewed the natural history of sickle cell disease in Uganda and found that pallor, jaundice and painful crises were among the most persistent features in all ages. It was found in Kenyan children with sickle cell anaemia that 25% presented with hand-foot syndrome, bone and abdominal pain, anaemia and

hepatosplenomegaly, which were among the commonest features (55). The same type of presentation has been reported in eastern Nigeria (52). In Sudan, patients were reported to present most commonly with vaso-occlusive crises, severe anaemia, hand-foot syndrome, fever and jaundice (13,41). The symptoms usually start after the age of 6 months but have been reported to present as early as 3 months after birth (41).

Vaso-occlusive episodes

The most prominent clinical feature of sickle cell anaemia is the painful crisis. It is often the first presenting complaint of the disease in the form of dactylitis, but it can occur in different organs, especially the long bones, abdomen and the chest. Painful crises show great heterogeneity among patients with sickle cell anaemia, extending from mild pain subsiding by itself to a severe agonising pain needing hospital admission and narcotic analgesics (115).

The pathophysiology of the vaso-occlusive phenomenon, the causative agent of the painful crisis, is largely unresolved (11,56). However, vaso-occlusion has been explained mostly by the abnormal shape and poor deformability of the sickle erythrocytes (42,121). Hebbel (42) and Kurantsin-Mills *et al* (59) have shown that increased adherence of the sickle cell to vascular endothelium is an additional cause of vaso-occlusion. Direct effects of the sickle cell on the endothelial cell by inhibiting DNA synthesis has also been reported (132). Platt *et al* (101), in their report of greater than 3000 patients in the co-operative study, showed that more than 40% of their SS patients did not seek attention for pain during 5 years of follow-up. They have also found that the rate of painful episodes varied directly with the level of haematocrit but inversely with the level of haemoglobin F. Powars (103) had earlier reported that patients with fetal haemoglobin levels of 20% or more had fewer painful episodes.

Infections in sickle cell disease

Patients with sickle cell disease have an increased susceptibility to bacterial infections, particularly to *Streptococcus pneumoniae*, *Haemophilus influenzae* (24,95,102), which cause fulminant meningitis and septicemia, and *Salmonella* (non typhi spp.) (135). It has been reported that patients with sickle cell anaemia are 600 times more likely to develop pneumococcal meningitis and 116 times more likely to develop meningitis due to *H. influenzae* type b (95). Powars and coworkers (102) have found that sickle cell anaemia

patients under the age of 5 years have 400 fold increased risk of developing pneumococcal meningitis and four fold higher risk of developing *H. influenzae* septicemia in those under 9 years of age. Recently it has been reported that patients under 5 years of age have 30-100 times increased risk of developing pneumococcal infections compared to healthy children of similar age (136). The increased risk of encapsulated bacterial infections in patients with sickle cell anaemia has mainly been attributed to functional asplenia (17,87,94,136). It has been reported that patients with sickle cell anaemia have reduced opsonic function against pneumococci (95,135). This has been explained by a defective alternative complement pathway (51). Wilson (134) and Chudwin *et al* (24) attributed the defective alternative pathway to a chronic activation of the complement system, while others relate it to antibody deficiency, specific IgG against the bacterial polysaccharide coat or specific IgG subclasses (18,82). Reduced concentrations of C3, C4 and factor B have also been reported (133). Other investigators, including ourselves, have not identified any defect in the complement activation (77,120,140,141). Complement proteins C3, C4 and factor B as well as the immunoglobulins IgG, IgA and IgM, have been reported to be normal and even high in patients with sickle cell disease (27,49,140,142,143).

Malaria

Malaria is a major disease of man. It has been estimated that more than 250 million are infected with malaria and 1-2 million die of it each year in Africa (47,114). The relationship of sickle cell gene and this deadly disease has been recognized for a long time (66). It has been postulated that the sickle cell mutation developed originally to protect people from dying of malaria (68). Evidence for the partial protection of sickle cell trait individuals from *Plasmodium falciparum* parasitaemia came from observations based on geographical distribution, population studies and hospital based patients studies (81). The mechanism of protection has been shown by *in vitro* experiments and *P. falciparum* cultures. The Plasmodium induces sickling of the erythrocytes containing Hb S and this will lead to either its direct damage, or because the cell will be removed by the reticuloendothelial system, breaking the life cycle of the parasite (37,67,68,81). Bayoumi (12) has suggested that the selective advantage of Hb AS individuals is due to earlier acquisition of immunity against *P. falciparum*. Other investigators have shown that individuals with sickle cell trait who have clinical malaria had lower plasma soluble IL-2 receptors and parasite counts compared to normal subjects (1). However, the ultimate mechanism is still not fully clarified. The

protective mechanism is true for the heterozygous individuals, but on the other hand, homozygous patients, although the SS haemoglobin does not favour the parasite growth, usually die of malaria in tropical Africa (87). Konotey-Ahulu (58) in Ghana has shown that 12.5% of his patients with Hb SS had crises precipitated by malaria. *Plasmodium falciparum* infection by itself is known to cause severe haemolytic anaemia (98). The additive effects of haemolysis with fever and vomiting, the common features of malaria, will be disastrous to an already frail patient (33).

Other complications in sickle cell disease

Complications are the sequelae of vaso-occlusion and anaemia. Essentially every organ in the body can be affected.

Splenic sequestration crisis. This is characterised by a rapidly enlarging spleen accompanied by a rapid fall of blood haemoglobin concentration (119) due to shunting of large amounts of blood in the sinusoids of the spleen (96). It is an acute medical emergency requiring urgent management with blood transfusion (91).

Liver dysfunction. The liver has been reported to show abnormal function tests due to sickling and infarctive phenomena. Gall stones were reported in more than 30% of patients over 10 years of age (119).

Kidney dysfunction. The kidney suffers multiple functional abnormalities and the well recognized one is the impaired ability to concentrate the urine, known as hyposthenuria (5,144). Even the heterozygous sickle cell individuals are known to suffer from this abnormality (14). Other abnormalities in the kidney include glomerular sclerosis, vascular congestion, edema, focal scarring and occasional papillary necrosis (144). It has been reported that 25% of patients with sickle cell disease have abnormal protein excretion in urine (32,65). End stage renal failure may ensue on these abnormalities (126).

Neurological involvement. The thromboembolic features of the disease may involve the brain and other parts of the nervous system producing different neurological deficits (93). Table I shows the different organs involved in sickle cell disease.

Table 1. Organ involvement in sickle cell anaemia and the pathophysiology of some of them.

Organ	Type of involvement	Pathophysiology
cardiovascular system	anaemia, cardiac enlargement, failure, infarction	haemolysis
		vaso-occlusion
lungs	acute pulmonary crisis, chronic pulmonary disease	vaso-occlusion infections
hepatobiliary	liver enlargement	
	gall stones	haeme-catabolism
	cirrhosis in adults	infarction
spleen	acute sequestration	
	infarctions	vaso-occlusion
	autosplenectomy	infarction
bone	aseptic necrosis (head of femur)	vaso-occlusion
	osteomyelitis	Salmonella
eyes	haemorrhage, neovascularization	vaso-occlusion
	rarely retinal detachment and loss of vision	
central nervous system	stroke, infarction, haemorrhage	vaso-occlusion
genitourinary	hyposthenuria, papillary infarction, nephrotic syndrome, focal sclerosis	vaso-occlusion
	priapism	vaso-occlusion
skin	leg ulcers (rare in Africa)	? vaso-occlusion

Laboratory variables

The diagnosis of sickle cell disease depends on the physical nature of sickle cell haemoglobin. Its gelation and distortion of the erythrocyte under low oxygen tension is used for the performance of the sickling test with 2% sodium metabissulfite as deoxygenating agent. Its reduced solubility when in the deoxygenated form is used for the solubility test. The charge difference on Hb S molecule is used for the diagnostic test, *viz.* haemoglobin electrophoresis, which was first performed by Pauling *et al* in 1949 (92). Haemoglobin electrophoresis is routinely carried out on cellulose acetate paper with Tris-EDTA-borate buffer at pH 8.4 or on acid agar with a citrate buffer, pH 6.0-6.2. Isoelectric focusing is another valuable tool. For the discrimination of the different genotypes, especially SS and

S-ß^{o}thalassaemia, measurement of Haemoglobin A_2 (HbA_2) concentration is needed. The HbA_2 concentration limit for SS is reported as 3.6% (113) but as high as 4.7% has been reported (109). DNA technology has been used for prenatal diagnosis in early pregnancy (6,53). Family study has a conclusive diagnostic value for SS haemoglobinopathy or S-ß^{o}thalassaemia (for details of diagnostic procedures, see 25&113).

A moderately severe anaemia is present by 6 to 9 months of age but can start earlier (69). Mean blood haemoglobin concentration has been reported as 75 g/L with a range of 55-95 g/L. In Kenya, haemoglobin levels of less than 80 g/L have been found in 85% of patients, with 10% having less than 50 g/L (55). The average haemoglobin levels in eastern Nigerian patients have been 72 g/L, ranging from 50 to 106 g/L (52). In eastern Saudi Arabia, the mean haemoglobin concentration has been 100 g/L (97). Reticulocyte count has been 8-12% but ranging from 5-30%, and 12-15 x10^9/L white cell counts (69).

Laboratory analysis among Sudanese patients has shown mean haemoglobin concentration of 73 g/L (13), ranging from 27 to 92 g/L (145), reticulocyte count of 15.1% and mean serum bilirubin of 36 μmol/L (13).

Erythrocyte membrane abnormalities

It has been shown that erythrocyte membranes from sickle cells have various abnormalities reflected in increased haemoglobin binding to the membrane, altered phospholipid asymmetry, decreased lipid fluidity, cellular dehydration, elevated intracellular calcium levels, altered membrane surface charge and defective cytoskeletal proteins. Asakura (8) has described increased denatured haemoglobin (hemichrome) SS binding to the erythrocyte membrane, which has been confirmed by other investigators using different methods (31,111). These hemichromes have been identified as the reagent responsible for the formation of reactive oxygen radicals at the membrane causing accelerated membrane senescence (44,60,107). The hemichromes firmly attached to the cell membrane also induce aggregation of band 3 protein within the membrane and to a lesser extent ankyrin and glycophorin (54,131). This aggregation forms a major site for autonomous IgG binding which may be a reason for the greatly shortened life span of sickle cells (45,54).

Phospholipids. The normal erythrocyte membrane lipid bilayer consists of approximately 27% phosphatidylcholine, 27% sphingomyelin, 29% phosphatidylethanolamine and 13% phosphatidylserine (128). All the sphingomyelin and 80% of phosphatidylcholine reside in

the outer leaflet and most of phosphatidylethanolamine and all of the phosphatidylserine are confined to the inner leaflet of the membrane (127). This asymmetrical distribution of the phospholipids is maintained by interaction of the lipids with the underlying cytoskeletal proteins and is ATP dependent (72). In the normal red cell and the oxygenated sickle cell there is low mobility of phosphatidylcholine, while in the deoxy-sickle cell this phenomenon is 4 times faster, and is completely reversible upon reoxygenation (35). Deoxygenation also exposes the aminophospho-lipids, phosphatidylserine and phosphatidylethanolamine, on the outer lipid bilayer leaflet (108). The increased transbilayer mobility of the phospholipids has been explained by the fact that haemoglobin polymers mechanically break the interaction of the phospholipid bilayer with the cytoskeletal proteins, herewith removing the constraint imposed by the latter (108,137). This is evidenced by the formation of spicules and vesicles, which do not contain spectrin, from the sickle cell membrane (4,36). The membrane-bound denatured haemoglobin has been recognized as the cause of increased rigidity of the sickled erythrocytes (31). However, decreased fluidity of the hydrophobic region of the erythrocyte membrane lipids has also been reported (106).

Sickled erythrocytes exhibit increased adherence to endothelial cells, monocytes and macrophages. The endothelial adherence is attributed to charge rearrangement, while adherence to monocytes is related to the exposure of the aminophospholipids in the outer membrane which contributes also to the thrombogenicity of sickled erythrocytes and vesicles released from them (45,138).

Cation imbalance. Tosteson *et al* (122) reported loss of potassium (K) from the cells during deoxygenation with a concurrent gain of sodium (Na). Later it has been reported that this alteration in cation permeability is dependent on ATP depletion, increased influx of calcium ions (Ca^{2+}) and deoxygenation. This leads to sickle cell dehydration and the formation of ISCs (40). It has also been recognized that ISCs have an elevated calcium content and accumulate Ca^{2+} during the sickling process (90). Other investigators have shown that Ca^{2+} accumulation does not activate the K channels in the deoxygenated sickle cells (19,89) due to the fact that the accumulated Ca^{2+} are internalized into inert endocytotic vesicles (20,63,80). Bookchin *et al* (21) have suggested that the dehydration effect of Ca^{2+} accumulation may be due to brief activation of the K channels of some SS cells.

Membrane proteins. The erythrocyte membrane proteins are identified as peripheral and integral proteins. The peripheral proteins include spectrin, ankyrin (band 2.1), band 4.1,

band 4.2, actin (band 5), glyceraldehyde 3-phosphate dehydrogenase (GAPDH, band 6), band 7 and band 8, while the integral proteins include band 3 protein and glycophorins A, and B (reviewed in 15). The nomenclature is based on SDS-PAGE mobility of these proteins described by Steck (116). These proteins lie in intimate contact with haemoglobin in the erythrocyte. In the sickle cell, defects have been reported in spectrin, ankyrin, band 3 and band 4.1 proteins (99). Investigations made on membrane proteins of sickle cells show no quantitative changes in most of these proteins (146). However, it has been reported that spectrin from sickle cells shows reduced phosphorylation whereas the phosphorylation of band 3 and other proteins are increased (29). Rank *et al* (105) have reported increased thiol oxidation of most of the proteins of the membrane which has been explained by the increased oxygen radical generation in the sickle cell membranes (44). It has also been shown that inside-out membrane vesicles from sickle cells exhibit reduced binding of radiolabeled spectrin (146), a defect which is exaggerated among ISCs. This failure to bind is related to defective ankyrin, which is also damaged by the oxygen radicals (reviewed in 99).

Band 3 protein is the major integral membrane protein, present in about 10^6 copies per cell. Apart from constituting the anion channel, band 3 has a cytoplasmic site for ankyrin binding which attaches it to spectrin and band 4.2 polypeptide. It also forms the binding site for several glycolytic enzymes, among which are the GAPDH and aldolase, and haemoglobin (reviewed in 15&117). Band 3 protein has been shown to manifest many defects, primarily due to its high affinity for haemoglobin (57,130) which exposes it to the oxidative radicals. A reduction in band 3 has been reported in the preliminary results of Platt (99), which is probably due to a mechanical removal of spectrin free vesicles from the sickle cells. The copolymerisation of band 3 protein, ankyrin and glycophorin with the denatured haemoglobin has been mentioned earlier.

The abnormalities of the erythrocyte membrane proteins certainly have their impact on the behaviour of the sickle erythrocyte and the clinical severity of sickle cell disease.

Trends in treatment and prognosis

Allogenic bone marrow graft is the only cure presently available for sickle cell anaemia. It is both full of risk and not available for most of the patients (147). The therapeutic trials so far have focused on the inhibition of Hb S gelation. Ethacrynic acid and its n-butylated derivative have been found to inhibit Hb S gelation in a pure haemoglobin solution, but

have been found to cause water loss and shrinkage of the erythrocytes (88). Ueno *et al* (124) have shown that methylacetate phosphate inhibits gelation in the intact cell by occupying the 2,3-bisphosphoglycerate binding cleft in the haemoglobin molecule and they also found that this substance reduces the density of sickle erythrocytes. Urea and cyanate have been tried only to be abandoned. An attempt to lessen the concentration of deoxy Hb S by the use of bicarbonate has not been successful. Substituted benzaldehydes have been found to stabilize the oxy-conformation of Hb S causing a left shift of the oxygen dissociation curve (148). Drugs have been used to improve the vaso-occlusive crises in the patients. Piracetam (39) and pentoxyfylline (70) have been tried with some effect.

High Hb F levels in patients with sickle cell anaemia have been found to be associated with mild clinical course of the disease, typically in eastern Saudi Arabia (97). Powars *et al* (103) have shown that Hb F levels of 10% have protective effect from end organ damage, while the painful crisis and splenic sequestration crisis will need 20% levels. Epidemiological, biophysical and pharmacological studies of patients with sickle cell anaemia by Nouguchi and coworkers (84) have shown that levels of Hb F of >20% will be needed to be protective from the severe complications of the disease. In Jamaica, a cohort study of 300 SS patients followed from birth, has shown that Hb F level was significantly related to dactylitis, painful crisis, acute chest syndrome and acute splenic sequestration, with high or moderate concentrations having an alleviating effect on these features (149). Azactidine (64) and hydroxyurea have been used to increase Hb F concentration (73,100). Charache *et al* (23) have reported the achievement of mean Hb F of 14.9% with a range from 1.9% to 26.3% by the use of oral daily doses of hydroxyurea for 16 weeks. However, they advised that this result is not conclusive clinically, as it is open ended.

Blood transfusion, analgesics and other supportive measures are all needed to alleviate the suffering of these patients. However, penicillin has been found to be life saving in patients under 5 years of age (100). Gaston *et al* (38), in an extensive cooperative study to evaluate prophylactic significance of oral penicillin, stopped the study eight months earlier due to the overwhelming benefit in the group taking daily oral penicillin.

Patients with sickle cell anaemia were known not to survive beyond their 20th birthday. However, improved understanding of the disease and more effective use of penicillin and antimalarial prophylaxis at an early age have made a remarkable improvement in the survival of patients with sickle cell disease (113). It has been reported that 50% of children with sickle cell anaemia in Zambia died before three years of age and only 10% of affected individuals in Zimbabwe were over ten years of age (69). In the United States, where

infections and acute splenic sequestration crisis could be controlled, the patients still die during young adult life due to progressive vasculopathy (104). While young children die of infections (62,104), the older individuals die of end organ failure. The improvement of this situation depends on the early identification of these individuals at increased risk of organ damage and giving them the appropriate management.

MATERIALS AND METHODS

Patients and controls

The subjects of the present investigation were 90 homozygous sickle cell anaemia (SS) patients, 27 heterozygous (AS) subjects and 28 healthy controls with normal adult haemoglobin (AA). Additionally 8 SS patients, 15 AS subjects and 8 controls were studied in another part of this work. All patients and healthy subjects were investigated after an informed consent in Sudan.

The patients' mean age was 6.1 years (range 0.4-22), 48 were boys and 42 girls. The AS subjects had a mean age of 10.3 years (0.3-24) and the AA controls 6.9 years (0.8-13). All the patients had complete clinical examination while only a personal history was obtained from the AS subjects and the AA controls.

Blood specimens were collected into sodium heparin tubes (Becton Dickinson, England) from each patient, healthy subject and control. Blood counts were done following standard manual methods (25), except blood haemoglobin estimation, which was done using a special haemoglobinometer (Hemocue, Helsingborg, Sweden). The blood samples were then centrifuged for 20 min at 3000rpm. The plasma was removed in plastic centrifuge tubes and converted into serum by adding a little thrombin. The serum was separated from the clot by centrifugation and stored frozen in liquid nitrogen until transported.

The red cells were washed three times with an isotonic phosphate buffer, pH 7.4, removing the buffy coat every time by suction. The cells were divided in 2 portions. One portion was used for preparation of haemolysates for electrophoresis and the other portion was stored in liquid nitrogen. The samples frozen in liquid nitrogen were transported in dry ice by air to Sweden. They were then transferred to a -70°C freezer and kept frozen until analysis. Diagnosis was made by haemoglobin elctrophoresis, Hb A2 level estimation and family study.

The serum was analysed by the routine methods used in the Clinical Chemistry laboratory at the University Hospital in Uppsala (74). S-5'nucleotidase (EC 3.1.3.5) was

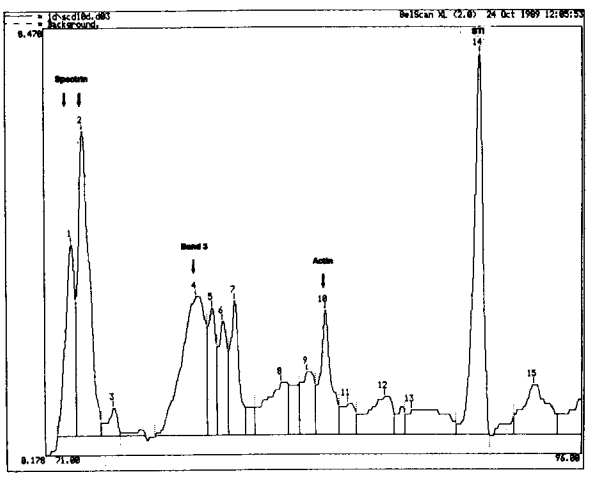
measured by a kit from Sigma (Sigma Diagnostics, New Jersey, USA). Strict quality control measures were observed.

Erythrocyte membrane preparation and electrophoresis (75). A method described by Dodge *et al* (28) was followed for preparing erythrocyte membranes with a modification as described in (75). Briefly, the frozen erythrocytes were thawn in cold isotonic phosphate buffer and immediately taken in haemolysing hypotonic buffer which contained 0.02% (v/v) Triton X-100. The haemolysate was centrifuged at 2000xg at 2°C using a Beckman ultracentrifuge (Beckman, USA). The pellet was washed three times with the same hypotonic buffer. The membrane was then stored at -70°C until used. The membrane preparation was thawn quickly in a water bath and solubilized in Tris-acetic acid buffer, pH 9.5, containing 2% SDS, 2mM EDTA, 1% dithiothreitol (DTT) and 0.005% bromphenol blue. A defined amount of soybean trypsin inhibitor, STI (22000 Dalton) was always included in the preparation and served as an internal standard for quantitative evaluation of the different protein bands in the electropherogram. The samples were run in Phast polyacrylamide gradient gels (Pharmacia Uppsala, Sweden), 8-25%. A low molecular weight kit (Pharmacia, Uppsala, Sweden) was run simultaneously for identification of different molecular weights. The gels were stained with Coomassie brilliant blue according to Heukeshoven and Dernick (48) and dried at room temperature. The gels were subsequently scanned using an LKB (LKB Bromma, Sweden) laser scanner. Figure 2 shows typical scans of a patient (A) and an AS subject (B). The different peak areas were related to the known internal standard with the formula a+b/b, a representing the respective protein peak and b the STI internal standard. This ratio is defined as the standardized peak area value.

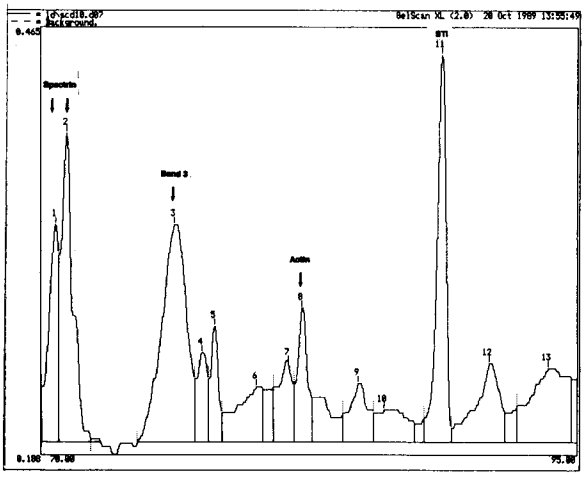
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) measurement in the erythrocyte membranes (76). GAPDH in the membranes was measured according to Bergmeyer (16) by following the conversion of NADH into NAD^+ at 340nm. The absorbance difference (ΔA_{340}) per min was measured from the linear initial part of the recording (Figure 3). Iodoacetamide showed specific dose-dependent inhibition of the enzyme indicative of the specificity of the reaction. The enzyme activity was expressed in μmol converted (NADH)/min/g membrane protein at 25°C.

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

2A



2B



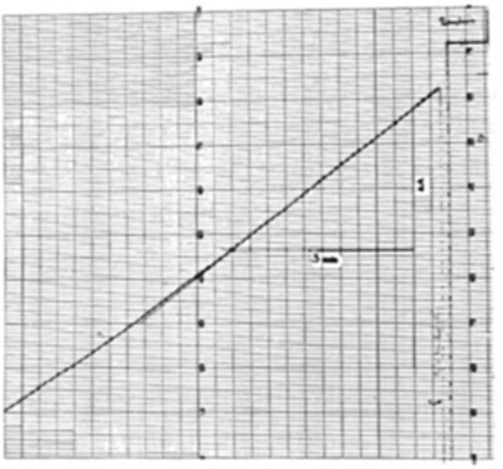


Figure 3. Linearity of membrane-bound GAPDH activity with time of a normal subject. Recording speed was 2 cm/min.

Complement and inflammatory marker study (77 and manuscript). The classical and alternative complement pathways were measured separately by the method of Nilsson and Nilsson (83). Complement proteins C3, C4, factor B and C3d together with the immunoglobulins IgG, IgM and IgA were measured by an automated immunochemical nephelometric method (Beckman Immunochemistry Systems, New Jersey, USA). Myeloperoxidase (MPO) and lysozyme concentrations in plasma were measured by a double antibody radioimmunoassay method (Kabi Pharmacia, Uppsala, Sweden).

RESULTS AND COMMENTS

Clinical findings, haematological and serum variables (74)

Ninety homozygous sickle cell anaemia patients, heterozygous (AS) subjects and controls (AA) from Sudan were investigated. Their clinical findings, haematological and serum variables were reported in this article. The patients from Sudan represented the severe type of the disease. Fifty percent of the patients presented with painful crises of the vaso-occlusive type, 50% of them (25% of all patients) first having dactylitis. At the time of

examination, 16% were found to have hand-foot syndrome and all were below 5 years of age. Fever was the next major presenting symptom in this group and was recorded in 47% of the patients. Table II shows the main clinical signs in the different age groups of the patients. Pallor indicative of anaemia had been a finding in almost every patient. However, only 17% of the patients had pallor as the main presenting problem. Enlargement of the liver was found in 60% of the patients while an enlarged spleen was only detected in 5 patients. Of the seven patients with pneumonia, 5 were under 5 years of age.

The patients had consistently low blood haemoglobin concentration, ranging from 31-94 g/L with a mean of 66 g/L. They showed marked reticulocytosis, with a mean of 20%, but ranging from 1-51%. The patients also showed significantly increased total white cell counts and the mean was 15.6 $X10^9$/L.

The biochemical tests of serum showed significantly increased levels of bilirubin (48 μmol/L), S-ASAT, S-GT (0.13-3.5 μkat/L) and S-urate (287 μmol/L, range 99-575). The patients also showed significantly lower levels of S-haptoglobin (0.24 g/L) and serum calcium compared to the AS subjects and the controls. The AS subjects had serum calcium level intermediate between the SS patients and the AA controls. The patients in general were not different from the others with regard to serum iron concentration. However, there were 14 patients (16%) with low serum iron indicating iron deficiency besides the haemolytic disorder.

Table II. Clinical signs in the different age groups of the SS patients.

Age (years) No.	<5 43	5-14 43	≥15 4	Total 90(%)
Pallor	42	41	4	87(97)
Jaundice	7	19	3	29(32)
Palpable liver	22	30	2	54(60)
Palpable spleen	1	4	-	5 (6)
Cardiac signs	5	17	2	24(27)
hand-foot syndrome	14	-	-	14(16)
Pneumonia	5	1	1	7 (8)
Osteomyelitis	-	2	-	2 (2)
Neurological signs	1	2	-	3 (3)
Scars of ulcers	-	1	3	4 (4)

Conclusion. Patients with sickle cell anaemia in Sudan show the severe type of presentation. Painful crises of the vaso-occlusive type followed by fever probably due to infections are the main problems among these patients and this is in agreement with other reports (13,41). Only 10% of the patients in this study were diagnosed as having pneumonia and osteomyelitis. The patients afflicted are usually under 5 years of age. Finally, the sickle cell haemoglobin may have a causal effect in the production of the relatively lower serum calcium levels, a phenomenon observed in a previous report (150).

Erythrocyte membrane proteins (75,76)

The erythrocyte membrane proteins were separated on SDS-PAGE and showed a subgroup of patients with lowered band 3 protein concentration on the electropherogram (Fig. 2A). The other subgroup had values comparable with AS subjects and the controls (75). This reduction in the first subgroup could not be related to other biochemical or clinical features of the disease. However, since band 3 is a major integral membrane protein with an important function in membrane integrity, its reduction in the membrane is expected to have a causative role in the process of haemolysis and the other membrane aberrations. In the subsequent article (76), we investigated the activity of glyceraldehyde 3-phosphate dehydrogenase in the erythrocyte membrane of 43 patients with homozygous sickle cell anaemia, 24 AS subjects and 27 AA controls. GAPDH is another membrane protein, comprising band 6 of Steck's nomenclature (15,116). On average, the patients showed a significant increase in activity compared to the AS and AA controls. However, they were again heterogeneous in this respect, as one subgroup of 15 patients showed a marked increase while the other subgroup was comparable to the controls and the AS subjects. The subgroup with the increased activity also had significantly lower blood haemoglobin concentration and higher S-LD compared to the subgroup with the normal levels. However, the subgroups did not differ in respect to reticulocyte counts or bilirubin and hence, the significance of this increase is difficult to interpret in conclusive terms and is invalid as an indicator of the severity of the haemolytic process in sickle cell disease. Neither did the subgroups differ in band 3 protein content which means that the subgroups with reduced band 3 protein (75) and increased membrane GAPDH (76) were not identical.

The conclusion from these two studies is that sickle cell anaemia has a heterogeneous membrane protein derangement. This aberration did not simply correlate to the disease

severity. This suggests a complex cause of the different features of sickle cell anaemia, with the membrane abnormality being part of it.

Complement and Neutrophil activation (77 and manuscript)

The subject of the increased bacterial infections in patients with sickle cell anaemia and its relation to a defect in the immunological system of these patients was addressed in ref.(77). Serum from 43 patients in the steady state as indicated by clinical examination and levels of C-reactive protein (CRP) < 10 μg/L was examined for the haemolytic activation of the classical and the alternative complement pathways. The levels of C3, C4, factor B and the immunoglobulins IgG, IgM and IgA were also measured. There was no difference in complement activation or the other serum variables between patients and the controls (Table III).

We performed also a preliminary study of the *in vivo* complement and neutrophil leucocyte activation by measuring C3d levels as well as the inflammatory markers, MPO and lysozyme, in EDTA plasma. The subjects of this study were 8 SS patients, 15 AS subjects and 8 controls. The results showed a significant increase of C3d and MPO but not lysozyme in the plasma of the patients compared with the AS subjects and AA controls. C3d was also higher among the AS subjects compared to the AA controls. This increase was not due to elevation of C3 and the ratio C3d/C3 did not essentially differ from the results obtained by observing C3d only. The C3d and MPO levels showed significant inverse correlations with haemoglobin in the SS + AS subjects, $r = -0.72$ and -0.68, respectively. MPO and C3d in plasma were significantly correlated ($r = 0.63$). These results indicate clear *in vivo* activation of complement and neutrophils but not macrophages in sickle cell anaemia patients.

The results from these studies show different conclusions. However, they are not contradictory. The *in vitro* measurement of the alternative complement pathway activity has rather poor sensitivity having a reference range from 50% to 150%. This method is clearly not capable of detecting minor changes, but since there was no reduction of the individual complement proteins, the process would be a compensated one. However, during increased demand the system may fail, having already exhausted its reserves.

Table III. Classical and alternative complement pathway activities, immunoglobulins IgG, IgA and IgM and complements C3, C4 and factor B (FB) levels in the plasma of SS patients (n=43), AS subjects (n=24) and AA controls (n=24).

	Classical pathway	Alternative pathway	IgG g/L	IgA g/L	IgM g/L	C3 g/L	C4 g/L	FB g/L
SS	69	130	16.6	2.10	1.41	1.10	0.22	0.44
	(20)	(33)	(4.6)	(1.30)	(0.74)	(0.172)	(0.058)	(0.08)
AS	73	120	14.5*	1.37*	1.45	1.14	0.23	0.43
	(18)	(36)	(3.3)	(0.41)	(0.74)	(0.172)	(0.055)	(0.097)
AA	64	120	16.1	1.57	1.48	1.08	0.22	0.41
	(22)	(34)	(3.5)	(0.42)	(0.50)	(0.195)	(0.059)	(0.10)

Mean and (SD) are given. *$p<0.05$. The other differences are not significant.

5'Nucleotidase (manuscript)

This paper investigated the levels of 5'nucleotidase (5'NT) in the sera of 59 patients, 17 AS individuals and 22 healthy controls. The mean concentration of S-5'NT was significantly higher in the patients compared to the controls. The AS subjects did not differ from either SS patients or AA controls and showed the tendency of being intermediate between the two groups. 5'NT concentration in serum from the patients showed significant correlation with S-GT, S-ASAT, S-bilirubin and S-ALAT, while it was not correlated with S-AP (Table IV). The results from this study suggest that the liver involvement in a subpopulation of patients with sickle cell disease is a mixture of hepatocyte damage and the biliary tree involvement.

Table IV. Correlation coefficients (r) on reciprocal comparisons between serum 5'nucleotidase (5'NT) and gammaglutamyl transferase (GT), bilirubin, aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT) and alkaline phosphatase (AP) in patients with homozygous sickle cell anaemia (n=59). The significance is given as p value. NS, not significant.

		S-GT	S-bilir	S-ASAT	S-ALAT	S-AP
S-5'NT	r	0.60	0.43	0.47	0.35	0.21
	p	<0.001	<0.001	<0.001	<0.01	NS

CONCLUDING REMARKS

The study of sickle cell anaemia patients from Sudan has elucidated several aspects of the disease.

The patients from Sudan have shown a severe type of disease with low haemoglobin concentration and various but heterogeneous aberrations in serum variables.Some patients had clear involvement of the liver, indicated by the extremely high serum levels of transferases, while other patients were in good shape, well adapted to the low haemoglobin concentrations. None of the patients had a high level of Hb F to warrant consideration of

a possible role for this feature. The discriminative factors may be other genetic conditions like α-thalassemia which has been shown to have alleviating effects on the disease (69) and which had not been excluded in this study. Socioeconomical status of the patients may also have an effect in the severity of the disease, an aspect which has not been investigated in this study.

The findings of the membrane protein aberrations represented by the reduction of band 3 protein in a subset of patients and the increase in GAPDH (band 6) in another subset warrant consideration in any future study design. At least the latter had shown a negative correlation with blood haemoglobin concentration and a direct one with S-LD, and the mean reticulocyte count in this subgroup had a tendency to be high, although not being significant. More extensive study of this phenomenon in an unbiased material would probably reveal the usefulness of the measurement of membrane GAPDH as an indicator for the severity of the haemolytic process in these patients.

The immunological problem in sickle cell anaemia seems to be a complex one involving not only discrete elements of aberrations but rather being multifactorial due to additive effects of minor deficits. Until this problem is solved, patients with sickle cell anaemia are recommended to have prompt and active treatment of every suspected infection with penicillin. The education of parents in this regard is of paramount importance for success, especially in the developing world.

The heterogeneity which has been observed in many aspects of the disease indicates that there is a subgrouping of patients in respect to the severity of the illness, probably more than 2 subgroups. The identification of these subgroups will be useful for the management of each individual patient.

Finally, the application of the new methods of study of the inflammatory process may represent new tools in the management of patients with sickle cell disease as they will identify the pathophysiological process in each patient.

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