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Ann V. Arthur
Yale University

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by

Ann V. Arthur

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ABSTRACT

ANALYSIS OF FETAL GLOBIN GENE EXPRESSION IN KUWAITIS WITH SICKLE CELL DISEASE. ANN V. ARTHUR, SECTION OF HEMATOLOGY, DEPARTMENT OF INTERNAL MEDICINE, YALE UNIVERSITY SCHOOL OF MEDICINE, NEW HAVEN, CONNECTICUT.

A clinically mild form of sickle cell disease, characterized by high hemoglobin F levels (in the range of 15-25%) is common in Kuwait. Studies have shown that selective regulation after gene expression of the two copies of the γ globin gene is one factor involved in controlling HbF persistence into adult life. The Aγ and Gγ globin genes of the β globin gene cluster are identical except at position 136 where either alanine (Aγ) or glycine (Gγ) is present. At birth, when HbF levels are relatively high, the Gγ: Aγ ratio is 7:3. As development proceeds, HbF levels decline sharply and the ratio changes to the adult value of 2:3.

In this study, we analyzed blood samples from 7 pedigree families (totalling 25 samples) and 6 unrelated individuals with various sickle cell syndromes. Hemoglobin lysates and globin preparations were electrophoresed on 12.5% polyacrylamide gels with 6M urea, 2% Triton X-100 in 5% acetic acid, to separate the β,α, Aγ and Gγ globin chains for analysis. HbF levels were determined using alkali denaturation.

Our results revealed an equal proportion of Gγ and Aγ globin chains and high HbF levels (ranging from 10.4%-60.7% in sickle cell patients), suggestive of a type of heterocellular persistence of fetal hemoglobin. However, neither Saudi Arabian or Kuwaiti sickle cell patients exhibit classical deletion or nondeletion HPFH, since parents of affected individuals did not have high HbF.

I. GENETIC BASIS OF SICKLE CELL DISEASE

Sickle hemoglobin ,HbS, is formed due to a simple point mutation in DNA in which thymine replaces an adenine residue in the sixth codon of exon 1 of the β globin gene. During subsequent protein synthesis that leads to the production of the β-globin chain, valine substitutes for glutamic acid in the sixth position.

Sickle cell anemia is a genetically inherited disease. Parents of affected individuals have either the heterozygous carrier condition known as sickle cell trait ,Hb AS, or are homozygous for sickle hemoglobin ,Hb SS.

The term sickle cell disease covers a spectrum of disorders ranging from the almost benign carrier state of sickle trait, to disorders of intermediate severity (Sickle β^{o}-thalassemia, Sickle β^{+}-thalassemia, Sickle α^{o}-thalassemia, Sickle α^{+}-thalassemia) to the most severe form - the SS phenotype. Also included in the category of sickle cell syndromes are Hb SC disease, Hb SD disease and Hb SO Arab disease. (16)

The increased clinical morbidity associated with these doubly heterozygous conditions compared to that seen with sickle cell trait is due to several factors. These factors include:

1) The presence of a second hemoglobin gene or mutant genes that leads to absent or reduced amounts of normal hemoglobin A. (i.e. Sickle β-thalassemia.)
2) The presence of a mutant β-chain that causes cellular dehydration and therefore increased intracellular hemoglobin S concentration. (i.e. Hb SC disease)

3) The presence of a hemoglobin that participates in polymerization to a greater degree than hemoglobin A does. (i.e. Hb SS and SO arab diseases) (33)

Epidemiological studies have shown that sickle cell syndromes have their highest prevalence in Black Africans and their descendents. However, the sickle gene has been found in Iran, Italy, Greece, Israel, India, Saudi Arabia and Kuwait.

The association between the sickle mutation and at least three separate restriction enzyme polymorphism haplotypes suggests that its distribution throughout the world is the result of multiple mutational events and dissemination via various trade routes. (13)

Geographically, the β^s gene is most concentrated in two areas of West Central Africa. One of these areas encompasses Nigeria and Ghana and the other Gabon and Zaire. It has been estimated that in these areas, as many as 25% of all newborns are AS heterozygotes. In African-Americans, the frequency of AS heterozygotes ranges from 7-10%. (16)

The sickle hemoglobin gene is also quite common in East Central India and in the north eastern region of Saudi Arabia (the Shia Oasis). Historical evidence suggests that the high prevalence of the β^s gene in Saudi Arabia and India may have arisen from migrations out of East Africa. African slaves were transported from East Africa to the Persian Gulf from 200-1500 A.D. There was also active slave trading to India from East Africa up until the 19th century. The frequency of the β^s gene in North, South, and Central America as well as the Caribbean is also linked to the slave trade that originated in West Africa. (16)

Evidence for several independent origins for the sickle cell mutation enhances epistasis (i.e. the effects of linked and unlinked genes on sickle gene expression) as demonstrated by hematological differences between SS individuals from Benin and Senegal that have different polymorphism haplotypes.(33) A map of the various β^s polymorphic sites has been deduced with the use of restriction enzymes. These enzymes recognize short, specific sequences of DNA for cleavage. The presence or absence of a particular site is denoted by a + or - sign. These haplotype maps allow investigators to refer to various polymorphisms via a simplified schematic representation. Figure 1 demonstrates these various haplotypes. A previous study describes the haplotype (----++-+) in chromosomes from individuals from Nigeria, the southwest of the Arabian peninsula, West Africa, Jamaica and the U.S. A different haplotype (++-++++-) not found in Africa, is seen in the eastern oases of Saudi Arabia and the west and east coast of India. The geographic distribution of the Asian β^s haplotype corresponds to the distribution of a clinically milder form of homozygous sickle cell disease associated with high levels of fetal hemoglobin (HbF). (51)

II. THE PATHOPHYSIOLOGY OF SICKLE CELL DISEASE

Under deoxygenated conditions such as those found in parts of the venous and capillary circulation, sickle hemoglobin undergoes polymer formation. NMR studies reveal that deoxygenated sickle cells become rigid even before they sickle and suggests that the accumulation of polymer leads to decreased cellular deformability and vaso-occlusion at precapillary sphincter levels. (33)

As the sickle red blood cells course through the circulatory system they undergo cellular dehydration and acquire a high MCHC and cell density. As sickle cells undergo deoxygenation they experience potassium leakage, and calcium influx and achieve a "sickle" shape. As this occurs, intracellular water is also lost in an attempt to reestablish osmotic balance. (33)

Several hypotheses have been formulated to explain the volume loss seen in sickle cells. They are as follows:

1) The phase transition of Hb S induces a direct volume difference between cells.
2) Hemoglobin gelation leads to decreased osmotic pressures.
3) Membrane stretching during sickling may lead to a disturbance in the cation homeostatic state.

Since HbS occupies the same volume in the gel and sol phases and the production of polymer and its effects on osmotic pressure do not sufficiently account for the volume loss seen, the later hypothesis involving membrane stretching appears to be the most scientifically sound of the three theories. (33)

In 1940, Drs. Ham and Castle proposed that a "vicious cycle of erythrostasis" explained the clinical nature of sickle cell disease.

They hypothesized that the dehydration and polymerization of hemoglobin S in sickle cells results in rheologic abnormalities that are thought to account for the vaso-occlusion, painful crises, tissue infarction and chronic hemolytic anemia seen in sickle cell patients. The rheologic impairment of sickle cells is due to:

1) Poor deformability of sickle cells.
2) Concentration of sickle cells in blood.
3) The increased adhesiveness of sickled cells to capillary endothelial cells.
4) The effects of sickled cells on whole blood viscosity.

Although the theory of the "vicious cycle of erythrostasis" remains to be proven scientifically, it has generally been accepted that vaso-occlusion is the main culprit responsible for the clinical manifestations of sickle cell disease.(33)

III. CLINICAL MANIFESTATIONS OF SICKLE CELL DISEASE

Individuals with sickle cell anemia are usually well at birth and become symptomatic between six months and twelve months of age. By ten to twelve weeks of age, red blood cell sickling, hemolytic anemia and increased levels of reticulocytosis becomes apparent in sickle cell patients. Infants with SS disease may appear clinically well since the major hemoglobin present at birth and for several months after birth is HbF. (33) In children, the clinical effects of sickle cell disease are usually acute and infectious in nature. However, adults with sickle cell disease tend to demonstrate chronic organ related damage from uncontrolled sickling. (78)

Studies designed by Scott et al., Ferguson et al., Porter et al., and others examined the clinical manifestations of sickle cell disease during the first year of life. Generalized dactylitis (hand-foot syndrome), infectious diseases and splenic sequestration crises were noted as major areas of clinical difficulty. (78)

Splenic sequestration crisis can be life threatening. During these episodes, children with progressive splenomegaly become severely anemic in the presence of actual or imminent shock. It has been theorized that the splenic sequestration crisis involves an accumulation of blood in the spleen. (16)

Pearson and O'Brien have found that sickle cell patients demonstrate changes in the reticuloendothelial function of the spleen during the first year of life. Children with sickle cell anemia often have splenomegaly by five to six months of age. As the spleen size increases, there is decreased phagocytic activity of the

reticuloendothelial cells in the spleen, demonstrated by isotopic techniques which reveal elevated numbers of "pocked" RBCs in the bloodstream. This leads one to conclude that children with sickle cell disease experience a type of functional asplenia as they mature. In African and American sickle cell patients it is typical to find that splenic infarction has occurred in older children (> 7 years of age) and adults. (78)

Children with sickle cell anemia have an increased susceptibility to contracting infectious diseases. Immunologic defects in the alternate complement pathway of sickle cell anemia patients leads to slow chemotaxis and inefficient phagocytosis by polymorphonuclear leukocytes. These factors as well as the functional asplenia exhibited by patients with sickle cell anemia most likely account for the increased prevalence of infectious processes in this group. (78) Incidents of sudden death in children with sickle cell anemia are often due to overwhelming pneumococcal septicemia. (78)

The sickle cell crisis is the hallmark of sickle cell disease and is characterized by excruciating pain in the back, extremities, chest and/or abdomen. Events that provoke painful crises still remain unclear; however these crises are often preceded by infection, dehydration, physical or emotional stress. (94)

The hemolytic anemia caused by Hb SS is usually moderate and often is overestimated due to plasma volume expansion. The anemia in and of itself is not a significant determinant of disease severity. The subsequent marrow expansion, hyperplasia of erythroid marrow and increased turnover of bile pigments (and its clinical

consequences of cholelithiasis) are problems encountered in all types of hemolytic anemias. With proper medical management these conditions can be compatible with a fairly normal, comfortable, long life. The vaso-occlusive effects of sickle cell anemia and subsequent damage to vital organs plays the major role in determining the degree of morbidity and mortality associated with the disease. (94)

The acute chest syndrome which presents with intense onset of fever, leukocytosis, pain on inspiration and expiration, dyspnea and cough is a common cause of hospitalization in sickle cell patients. Methods to accurately distinguish between micro-infarctive events and infectious processes in the pulmonary system have yet to be developed. (16)

Patients with sickle cell disease may also experience neurological deficits and seizures as a result of a cerebrovascular accident. Children with sickle cell disease also have delayed growth and development. Adults with sickle cell anemia commonly exhibit chronic organ damage that is most pronounced in the spleen, heart, lungs, kidneys, eyes and bones.

The major renal complications of sickle cell disease are uremia, hyposthenuria, nephrotic syndrome, and hematuria. (77) Microinfarction of the renal papillae can cause a loss of the ability to concentrate and dilute urine. This isosthenuria in turn leads to zinc deficiency and wasting. (10) Such organ system failure can in turn become life threatening.

The lifetime prognosis of patients of sickle cell disease in the U.S. is not known. In 1970, Scott calculated a median expected

survival of 20 years of age using data from the U.S. Bureau of Vital Statistics. Today, therapeutic interventions aimed at preventing dehydration, maintaining fluid balance and halting the spread of infections has not made it unusual for sickle cell patients in industrialized nations to survive into and beyond the third and fourth decades. (16)

IV. CLINICAL HETEROGENEITY IN THE SICKLE CELL SYNDROMES

One of the most striking features about sickle cell anemia is the broad spectrum of clinical severity seen in affected patients. For example, some patients are incapacitated by recurrent episodes of painful crises and ensuing organ damage while other patients with the same apparent phenotypic expression of HbS disease, have, with the exception of their hemolytic anemia only mild and inconsequential clinical sequela. (94) The co-inheritance of a gene for α-thalassemia or elevated levels of HbF (i.e. hereditary persistence of fetal hemoglobin - HPFH) have been proposed as factors modulating the clinical severity of sickle cell anemia.

Thalassemia is a genetic disorder that leads to a defect in the production of one or more of the globin polypeptide chains of hemoglobin. This defect in turn leads to absent or decreased synthesis of the affected chain. The red blood cells of affected individuals tends to be hypochromic (low intracellular hemoglobin concentration) and microcytic (smaller than normal).

Co-inheritance of a gene for HbS and for β-thalassemia sometimes results in a milder clinical course than that experienced by patients with the SS phenotype. (16) The effects of α-thalassemia on disease severity in sickle cell anemia remains uncertain. It has been suggested that the effects of hypochromia on viscosity of blood or the diminution of internal viscosity of the red blood cells as a result of decreased mean HbS concentration are possible means by which mild symptoms of HbSS are produced. (32)

Alpha-thalassemia is made up of four "classic" syndromes:

1) Alpha-thalassemia-2-trait is very common in Blacks, (with a frequency of 15-20% in some populations). In this condition, all four α globin gene loci fail to operate correctly.

2) Alpha-thalassemia-1-trait is a condition in which there are two dysfunctional gene loci.

3) In HbH disease three dysfunctional α globin gene loci are present.

4) In Hb Bart's, hydrops fetalis is often seen, as a result of a defect in all four alpha globin gene loci.

Although the above syndromes are classically caused by deletion of one, two, three or all four of the α globin genes, non-deletion forms of α-thalassemia also occur. (10)

The effect of α-thalassemia on disease severity of sickle cell anemia remains unproven. One study performed by Dr. Mears and his colleagues provided for the analysis of the alpha gene deletions by restriction endonuclease mapping of DNA from Black Americans and Africans. It was demonstrated that subjects with sickle cell anemia possess chromosomes bearing α gene deletions in greater numbers than normals or individuals with sickle trait. They also concluded that α gene deletions that cause α-thalassemia allow for prolonged survival in individuals with sickle cell anemia. Higgs et al., were unable to demonstrate increased survival associated with co-inheritance of HbS and α-thalassemia. (40)

However, the investigators note that it is not clear if the co-inheritance of sickle cell anemia and α-thalassemia trait leads to a milder clinical course (fewer and less severe crises) in affected individuals. (56)

In hereditary persistence of fetal hemoglobin (HPFH), morphologically normal red blood cells in individuals who are heterozygous for the causative mutation can have HbF levels as high as 20-30%. Individuals who are homozygous for HPFH can produce up to 100% HbF. In 1961, Jackson et al. evaluated sixty-one patients with sickle cell anemia for clinical severity. Using a pathologic index rating, they found that 38 patients whose erythrocytes contained 12% or more HbF had a significantly lower pathologic index rating compared to 23 patients whose red blood cells contained little HbF. They concluded that the increased amounts of HbF protected red blood cells from sickling and thus decreased the degree of severity of sickle cell anemia. (46)

V. HEMOGLOBIN SWITCHING

The mechanism by which red blood cells switch from synthesis of HbF to HbA during the neonatal period had been the subject of much study and interest. By clarifying the means by which hemoglobin switching occurs, we may be able to elucidate the developmental regulation of gene expression as well as determine means of reactivating or preventing suppression of gamma globin gene expression. As elevated HbF levels have been linked to milder clinical courses in sickle cell disease and thalassemia, a potential form of gene or pharmacological therapy may be developed based on an understanding of hemoglobin switching.

During the last six months of gestation, fetal red blood cells contain mostly HbF. As the first year of life progresses, the infant's red blood cells contain predominantly HbA. Adult red blood cells contain mostly HbA with small amounts of HbF and HbA_2. (92)

Figure 2 is a schematic representation of the human globin gene clusters and an indication of when particular hemoglobin types are produced in the course of human development. HbF is composed of two gamma chains and two alpha chains. There exists two γ globin genes, Gγ and Aγ. The Gγ gene encodes for glycine at position 136 and the Aγ gene encodes for an alanine residue at this position. During fetal development, the Gγ to Aγ ratio is 3:1 in RBCs. As the hemoglobin switch occurs in the perinatal period leading to increased synthesis of HbF the Gγ to Aγ ratio changes from 2:3 (the ratio seen in adult RBCs) (92)

The exact mechanism that regulates the normal switch from γ to β globin gene expression remains unknown. However, it appears

that the switch is controlled more by a developmental clock and gestational age than by intrauterine or environmental factors, as prematurity has little effect on the process. (16) Some modulation of the process can occur as in intrauterine growth retardation (IUGR), maternal anoxia, and hyperinsulinism secondary to maternal diabetes mellitus in which there seems to be some delay in the switch. Also, in some cases of Down's syndrome (Trisomy 21) and the C/D chromosomal translocation syndrome, there appears to be an acceleration of the hemoglobin switch. (16)

Several hypotheses exist for the mechanism of the fetal switch:

-Clonal model: hemoglobin switch occurs due to a progressive replacement of cells derived from a fetal stem cell lineage by cells derived from an adult stem cell lineage.

-Adult vs. fetal erythroid cell expression is regulated by changes in the degree of DNA hypomethylation and DNAase I hypersensitivity of the 5' flanking region of DNA of both the gamma and beta globin genes.

-Regulatory DNA sequences on the inter- γ/δ gene region may be responsible for the fetal to adult hemoglobin switch. (16)

During fetal development, the γ and β genes are expressed simultaneously. At the beginning of the liver stage of erythropoiesis (weeks 5-20 of gestation), the γ gene is approximately fifty times more active than the β gene. By week 30-33, β chain synthesis slowly increases to approximately ten percent of γ chain synthesis. As the rate of γ chain synthesis falls,

the synthesis of β chains increases dramatically. At birth, the normal infant has 60-80% HbF in its RBCs. By 16-20 weeks of age, HbF levels are approximately 3%. By age two years, HbF levels are usually that of adult RBCs - < 1%. (91) In adults, hemoglobin F is found in only a subpopulation of RBCs (usually <8%) referred to as F-cells. While F-cells appear to express other features of fetal red blood cells, they are distinct form true fetal red cells. (102)

Various mutations have been associated with increased levels of HbF. In deletion mutations, there is deletion of a portion of the β globin gene cluster. Non-deletion mutations reflect point mutations within or outside of the cluster. Both types of mutations can cause a uniform distribution of HbF (pancellular) or a nonuniform distribution (heterocellular).

In heterocellular distribution of HbF, mutations can increase the percentage of F-cells and/or increase the average amount of HbF per F-cell. These mutations can increase Gγ globin gene expression only, Aγ globin gene expression only or both. Thalassemia mutations can also lead to the production of hypochromic, microcytic RBCs with increased HbF production. When the mutation leads to morphologically normal cells associated with increased HbF levels in heterozygous individuals, the condition is known as hereditary persistence of fetal hemoglobin (HPFH). (92)

Increased production of HbF can also be observed during episodes of erythropoietic stress (as in recovery from chemotherapy, bone marrow transplantation and chronic congenital hemolytic anemias). For example, acute hemolysis results in increased F-cell production, although required or congenital chronic

hemolytic anemias are rarely associated with significant elevation in HbF levels. Also, in severe untreated iron deficiency the F-cell levels is within normal limits. After iron replacement there is a marked increase in reticulocyte response which in turn leads to erythroid expansion and the preferential production of F-cells. How accelerated erythropoiesis leads to increased F-cell production remains a mystery. One theory is that during erythropoietic stress there is an increased chance of premature commitment of early erythrocyte progenitors which can lead to an increase in F-cell production. (92) In support of this idea is the observation that in acutely anemic baboons F-reticulocyte levels are low. After a few days the F-reticulocyte levels increase. When the baboon becomes chronically anemic the proportion of F-reticulocytes decrease despite persistent reticulocytosis. This is thought to occur due to the expansion of the erythron which allows reticulocytosis to occur with less disruption of erythropoiesis and subsequently less probability of increased F-cell formation. (92)

Demonstrated stimulation of HbF synthesis in vitro using bone marrow cultures from adults by Papayannopoulou et al. indicates that the HbF to HbA switch is reversible and that induction of fetal hemoglobin synthesis in the adult is possible. By using antibodies and immunofluorescent labeling these investigators were able to gather evidence for a cellular restriction of HbF. In a given HbF colony, all the cells reacted to fluorescent antibody with the same degree of intensity regardless of colony size. It thus seems probable that all the cells were programmed to have similar levels of γ chain formation. This suggests that the signal for turning on γ

chain synthesis is present on the original stem cells from which colonies are derived. (68)

The premise that γ gene expression is dependent on erythroid stem cell differentiation and is characteristic of early erythroid cells was further supported by a study by TH. Papayannopoulou et al. in which erythroid colonies derived from primitive erythroid precursors demonstrated consistent activation of HbF synthesis. It appears that this property is lost when erythroid precursors of the adult further differentiate in vivo. These findings suggest that the use of erythroid progenitor cells may provide a useful, early accessible model for studying the regulation of globin gene expression. (70)

VI. SICKLE CELL DISEASE IN SAUDI ARABIA

Dr. H. Lehman and associates were the first to describe the high prevalence of the sickle cell gene in Saudi Arabia. Their analysis of more than 1000 blood samples from employees of the Arab-American Oil Company (ARAMCO) in Danman revealed that 10-25% of the people residing in the eastern province of Saudi Arabia had sickle cell trait. (53)

Sickle cell disease in the eastern province of Saudi Arabia is characterized by a relatively mild clinical course which appears to be due to the elevated HbF levels seen in this population. It has been suggested that this group of patients with mild sickle cell disease may serve as a model for understanding what clinical sequela may be ameliorated via manipulation of HbF production after birth.

In 1979, Perrine et al. described the presence of mean HbF levels of 22-26% in Saudi Arabian children and adults. They also demonstrated the presence of higher levels of HbF during the first few years of life in these patients. They found a mean level of HbF of 32%, even after the age of three. In contrast, in most other sickle cell populations, HbF levels fall more slowly after birth than normally but tends to be less than 15% by age one and less than 9% by age three. Forty-two patients with sickle cell disease were studied along with matched controls, for clinical complications specific for sickle cell disease. They found that vaso-occlusive crises were rare and generally slight in nature. Two patients suffered from hand-foot syndrome. Hypoplastic crises in one patient were severe enough to warrant blood transfusions. There was no

were severe enough to warrant blood transfusions. There was no case of acute splenic sequestration crisis. One patient had osteomyelitis and one had salmonella septicemia. Fourteen of the 42 patients with sickle cell disease were hospitalized during the first three years of life compared to 6 of the controls. The most common causes of hospitalization in the sickle cell patients were respiratory infections and gastroenteritis. Clinical assessment of growth and development were within normal ranges and did not show significant differences from controls. There were two deaths reported. One child died apparently from septicemia at age 25 months. Another child died at age 35 months from what probably was gastroenteritis. (75)

Of interest is the apparent preservation of splenic function in Saudi Arabian children with sickle cell disease. The presence of Howell-Jolly bodies is thought to be a reliable indicator of loss of splenic function. Only one-sixth of Saudi infants with HbSS had Howell-Jolly bodies when studied at 18 months and older (mean age of 44 months). After age one, patients of African descent with HbSS were reported to have lost their splenic function in two-thirds to four-fifths of cases. (75) This fact may help explain the difference in mortality rates during the first two years of life: reported as 13% for Jamaicans and zero for the Saudi Arabians with sickle cell disease. (75)

Saudi Arabians with HbSS are 34 times more likely to get pneumococcal meningitis, and 8 times more likely to get bacterial meningitis than the general population. However, Blacks in Miami

with HbSS have a relative risk of 309 for all bacterial meningitis and 579 for pneumococcal meningitis. (74)

When the mortality rates of 216 Saudi Arabs was compared with that of 207 Chicago Blacks they were found to be 0.9% in eight years and 9.1% in five years respectively. Sickling related complications occurred less often than seen in comparable groups of Blacks with HbSS from the U.S. or Caribbean. For example, none of the Saudi Arabians studied by Perrine and his associates in 1978 had a history of leg ulcers while 44% of the American or Jamaican Blacks did. Eight percent of the Blacks age 10-19 had femoral head necrosis compared to 4.4% of the Saudi Arabians in the same age group. Six percent of Blacks had a history of pneumococcal meningitis and 10% had a history of bacterial meningitis. In contrast, 1.5% of Saudi Arabs had pneumococcal meningitis and 2.6% had bacterial meningitis. (74)

As in American, African and Caribbean Blacks, a wide spectrum of clinical severity is seen in Saudi Arabians with sickle cell disease. However, the overall clinical course of the disease is milder. Death was found to be rare in early childhood. The few deaths that have occurred in adolescents and adults from infectious causes may reflect slower pathological damage to vital organs; especially delayed splenic atrophy. Many patients have splenomegaly into adult life - a factor which may or may not be influencing susceptibility to infection. (74)

M.A.F El-Hazmi et al. in their study of Saudi Arabians with Hb SS found that some patients with low HbF levels have mild symptoms, while others with high HbF have more severe clinical

manifestations, indicating that some other factors are involved in ameliorating sickle cell disease in Saudi Arabians. (32)

Karim Kamel studied 11 patients with homozygous sickle cell anemia who were non-Sheeah Arabs from the south eastern coast of the Arabian peninsula and discovered that they had elevated HbF levels ranging from 8-18%, with a mean of 11.9%. However, he found no significant correlation between a patient's HbF level and the patient's clinical manifestations. He proposes that intermixing of the African sickle gene in this population is a possible explanation for the clinical diversity observed. (47)

El-Hazmi also studied sickle cell disease in Arabia and found elevated levels of HbF. The HbF was unevenly distributed among red blood cells (heterocellular distribution). He proposed that acquired anemias such as iron deficiency or pernicious anemias may lead to low hemoglobin concentrations that produce a new steady state in which there are less frequent painful crises occurring. (30) Thus, genetic and acquired conditions may allow for the clinical diversity seen in sickle cell disease.

An examination of 53 children from the Saudi-Arabian south western province with sickle cell disease by El-Hazmi et al. revealed some interesting findings. HbF distribution revealed marked variation from 1.0% to 25% with a mean of 10%. Hematological parameters did not show any statistically significant differences between subgroups with low HbF (less than 5%, 5-10%) and high (greater than 10%) HbF. This suggests that the HbF level is not the only factor influencing hematological parameters in patients with sickle cell disease from the south western province of Saudi

Arabia. Patients also demonstrated a high frequency of co-inheritance of alpha and beta-thalassemia, and G-6PD deficiency. It was found that total hemoglobin, RBC count and PCV had a higher mean value in sickle cell patients with alpha or beta thalassemia compared to those without thalassemia. Red cell indices (MCV, MCHC, MCH) were significantly lower in sickle-thalassemia patients but fetal hemoglobin levels did not show any statistical significance. (31)

Clinical manifestations of sickle cell disease in the south western province of Saudi Arabia were found to be severe. All of the children were anemic. 85.4% had received blood transfusions and of these 43.9% had several blood transfusions. Seventy-eight percent demonstrated splenomegaly and 65.8% had hepatomegaly. One patient had undergone splenectomy. (31)

A new finding in sickle cell disease patients in Saudi Arabia was the presence of hand-foot syndrome in 34.1% of the children studied from the south-western province. Vaso-occlusive crises were fairly common, occurring in 75.6% of those studied. Hemolytic crises were seen in 19.5% and infective crises in 9.7% of those studied. Interestingly, the presence of high HbF levels, alpha or beta thalassemia or G-6PD deficiency did not appear to ameliorate the clinical manifestations of sickle cell disease in the same way they appear to in the sickle cell population in the eastern province; although co-inheritance of alpha or beta thalassemia did improve hematological parameters. (31)

The presence of a clinically severe form of sickle cell disease in the Saudis that resembles the clinical course in Black Africans and Americans suggests the presence of a different sickle gene in these patients or the possibility that genetic and/or environmental factors that help produce a mild sickle cell disease in the eastern province are absent in the south western province of Saudi Arabia. (31)

Red blood cells in patients with sickle cell disease, that have increased amounts of HbF tend to have increase survival rates. It has been suggested that the lower levels of HbF (usually 5-10%) that occurs in Blacks with HbSS is probably due to the differential survival of cells with HbF, rather than an actual increase in HbF synthesis. (74)

Nonetheless, attempts to draw connections between clinical severity and HbF levels have led to ambiguous results because it is difficult to objectively define clinical severity and other genetic, environmental and physiological factors affecting clinical status .

Wood et al., studied HbF synthesis in Saudi Arabian and African sickle cell patients in an attempt to determine if the increased level of HbF seen in the former group was due to the selective survival of cells with HbF or a true increase in γ chain production.

Experiments conducted in vitro and in vivo have shown that cells containing HbF have a longer survival in the circulation than cells without HbF. This is thought to be due to the inhibitory effect that HbF has on the sickling process of HbS molecules leading to a reduction in the number of red blood cells that become irreversibly sickled cells. (16)

Using single cell immunodiffusion, Dover et al. found no significant correlation between the amount of HbF/F-cell and the number of F-cells. Thus, the amount of HbF/F-cell may be an independent variable which also contributes to the overall levels of HbF. (28) Later studies revealed that the synthesis of γ chains was considerably lower than the levels of HbF in African and Saudi Arabian cases; suggesting an increased role for the selective survival of F-cells in sustaining increased HbF levels. (102)

In 1987, M.A.F. El-Hazmi and associates studied 285 males and 432 females from Al-Hafout, Al-Qateef and neighboring villages in the eastern province of Saudi Arabia. In the 55 SS homozygotes studied there were no cases of leg ulceration, or hand-foot syndrome. Abdominal pain and pain in the bones and joints appeared to be one common complaint. Almost 70% of the SS patients had received blood transfusions although actual sickle cell crises were noted as being "rare." Fifty-six percent had anemia, 36% had hepatomegaly, 45% had splenomegaly and osteomyelitis was seen in 45% of the cases studied. The data for the individuals homozygous for the sickle cell gene revealed variation in the clinical and hematological data; some individuals exhibited chronic anemia and others almost normal values for Hb, RBC and PCV. Co-inheritance of α-thalassemia has been suggested as a possible modifier of the clinical sequela of sickle cell disease. The theory is that hypochromia may cause a decrease in viscosity of blood or in the internal viscosity of the red cell due to decreased mean cell Hb SS concentration and subsequently resulting in decreased intravascular sickling and mild symptoms. (32)

Pembrey et al. measured HbF levels in 137 normal individuals, 109 sickle trait individuals and 237 individuals homozygous for sickle cell disease. The HbF found had a heterocellular distribution. (73)

Using the premise that the level of HbF in the peripheral blood is a result of the rate of HbF synthesis and differential destruction of F-cells and non F-cells, investigators sought to evaluate the roles of coexistent α-thalassemia and/or G-6PD deficiency in increasing HbF levels. If the high levels of HbF in Saudi Arabs with sickle cell disease was due largely to the concentration of F-cells in the peripheral blood there would exist a positive correlation between the rate of hemolysis and HbF level. The opposite was shown - an inverse correlation between HbF level and the reticulocyte count. Thus, the increased level of HbF was thought to be primarily due to increased synthesis of HbF which in turn decreased hemolysis by inhibiting cell sickling. Alpha-thalassemia and G-6PD deficiency were also shown to have little effect on promoting high levels of HbF (73)

The heterocellular distribution of HbF and low levels of HbF found in normal (AA) individuals and individuals with sickle cell trait (AS) excludes the pancellular, Negro and Greek forms of HPFH as possible explanations for the increased levels of HbF seen in Saudi Arabians with sickle cell disease. Population surveys have revealed little difference in HbF levels in normal and sickle trait individuals of Arabian or African descent. Low levels of HbF in normal and AS individuals excludes the possibility of a co-dominant

heterocellular HPFH gene in linkage disequilibrium with the sickle gene. (73)

Another possibility is that there is present a truly recessive HPFH gene which is in linkage disequilibrium with the sickle gene. This hypothesis would prove to be true if individuals from the Saudi Arabian oasis population with sickle β^o-thalassemia have similar high levels of HbF. (72)

Using a radioimmunoassay to assess the amount of HbF produced in vitro by erythroid progenitor-derived erythroblasts, investigators have sought to determine the basis of increased HbF. Results of this study revealed that HbF synthesis is a major contributor to increased HbF levels in Saudi Arabians with sickle cell anemia. The findings also suggest that the high HbF levels are inherited from at least one parent but its expression requires increased erythropoiesis. The Saudi Arabian patients studied consistently had an increased ratio of $G\gamma$ to $A\gamma$ globin. In contrast, 80% of Black Americans with sickle cell disease have 40% $G\gamma$ and 60% $A\gamma$ with only 20% having more $G\gamma$ than $A\gamma$. (59)

Interestingly, Miller and her colleagues found that patients homozygous or heterozygous for the ++-++ haplotype and with the SS phenotype have an increased level of HbF synthesis. However, homozygosity for the ++-++ haplotype in and of itself did not appear to control HbF levels. Individuals heterozygous for the sickle gene and homozygous for ++-++ had only modest elevations in HbF. (58)

Miller proposes that the amount of HbF produced depends on erythropoietic stress, the degree of maturation of the progenitor cells and the "permissiveness" of the γ globin gene.

In a follow-up study, Miller studied 55 members of five families with sickle cell disease to determine if the specific γβ gene cluster seen in these individuals could explain the increased HbF levels, increased Gγ/Aγ ratio and elevated HbF production by progenitor derived erythroblasts. Starting with the knowledge that most Saudis with sickle cell anemia were homozygous for the haplotype ++-++++- characterizing the β globin gene cluster, the γ globin gene region was isolated via molecular cloning. The nucleotide substitution C-> T at position -158 5' to the Gγ cap site was recognized using restriction endonucleases. The results revealed that Saudis homozygous or heterozygous for the sickle gene with a -158 C->T substitution, even on one chromosome, had an elevated level of Gγ > 50%. No significant differences were found between heterozygotes or homozygotes with the C->T substitution on one or both chromosomes. This suggests that while an increase in Gγ globin may be associated with the C->T substitution it does not necessarily produce a measurable increase in total HbF production. (58)

Miller also found that Saudis with one copy of the "common" eastern oasis haplotype +++-S do not have elevated total circulating HbF (in contrast to their African and Mediterranean counterparts) but did demonstrate variable increases in the Gγ /Aγ ratio ratio. The authors confirmed their previous finding that the presence of the +++-S γβ haplotype on both chromosomes correlated with the extent of HbF production in Saudis. This association does not suggest a tight linkage. The authors hypothesize that the -158 C->T

substitution may increase the ability of the Gγ region to respond to transacting proteins. The actual amount of the HbF produced in red blood cells may be dependent upon the intracellular level of these transacting proteins and on the specific DNA sequences near and/or far away from the γ globin genes. Also, erythropoietic stress may affect the transacting regulatory protein levels and lead to increased expression of Gγ genes and total HbF. (58) The identification of the regulatory proteins controlling globin gene expression and description of their function will help to clarify factors responsible for the HbF levels in Saudi Arabians and others with sickle cell anemia.

VII. SICKLE CELL DISEASE IN KUWAIT

The high frequency of the sickle gene in the region bordering the western coast of the Arabian gulf appears to be due to three factors:

1) Falciparum malaria was endemic in this area into the mid-20th century, allowing for a selective survival advantage to heterozygotes.
2) Homozygous disease appears to be compatible with survival into adult life and reproduction.
3) A high rate of consanguinity exists with frequent first cousin marriages). (71)

Of the Kuwaiti patients studied by Pearson et al., 47 were found to have Hb SS disease while 21 had Hb Sβ-thalassemia. No significant difference was found between the Kuwaiti Hb SS patients and the Hb Sβ-thalassemia patients in regards to hemoglobin level, reticulocyte count, and percent HbF. This is similar to the situation seen in the U.S., where Sβ-thalassemia and SS patients have similar clinical and hematological pictures. (71)

Kuwaiti sickle cell patients were compared with American sickle cell patients and the former group was found to have higher levels of HbF than the latter group. Hemoglobin levels were significantly higher and reticulocyte counts significantly lower in Kuwaiti patients. (71) Hemoglobin levels in the Kuwaiti sickle cell disease patients ranged from 70-120 g/L. None of their American counterparts had hemoglobin levels above 90 g/L or HbF levels over 10%. (71)

Kleinhauer-Betke preparations were done on six Hb SS and four Hb Sβ-thalassemia Kuwaiti patients and all revealed a heterogeneous distribution of HbF in their erythrocytes; a finding similar to that seen in Saudi Arabians with sickle cell disease. (71)

Analysis of clinical records of Kuwaiti patients by Pearson et al., revealed a relatively mild clinical course for most. Only 10% had experienced sickle cell dactylitis (hand-foot syndrome) compared to one-third of the American cases. Painful crises appeared to be more responsive to analgesia and IV hydration than American cases. There were no new episodes of splenic sequestration syndrome and only one episode of stroke and two episodes of Salmonella osteomyelitis at the Sabah Children's Hospital between 1976-1985. (71)

Eighteen of 21 patients with Sβ-thalassemia and 13 out of 47 with SS disease had splenomegaly. No American sickle cell patients had palpable splenomegaly. Thirty-five percent of Kuwaiti patients with Sβ-thalassemia had weights < 10th percentile and 75% of Kuwaiti HbSS patients had weights at this level. In the American group, 35% showed this degree of growth delay. (71)

During the years 1976-1983, only one Kuwaiti sickle cell patient died. This death was determined to be due to causes unrelated to sickle cell disease. There were no recognized causes of overwhelming sepsis, which is seen in 10-20% of patients in the U.S. The lack of severe infectious complications in Kuwaiti sickle cell patients may be due to the overall preservation of splenic function. (71)

Pearson et al., evaluated this premise by analyzing the amount of pocked RBCs found in Kuwaiti versus American sickle cell disease patients. Preservation of splenic function associated with a low percentage of pocked RBCs in sickle cell patients from the eastern province of Saudi Arabia has been reported. Pearson et al., found that in 10 Kuwaiti patients with HbF levels > 15% and concomitant Hb SS or Hb Sβ-thalassemia, all had normal splenic function. Of note, all the American cases had >10% pocked RBCs indicating functional hyposplenism. (71)

A third of patients with a hemoglobin SF electrophoretic pattern (21/68) were shown by family studies to have the Sβ-thalassemia genotype. It appears that the α-thalassemia gene may also be prevalent in Kuwait as some of the individuals with homozygous sickle cell disease had microcytosis. (71)

American sickle cell patients have a greater degree of hemolysis than their Kuwaiti counterparts as demonstrated by their lower mean hemoglobin levels and higher reticulocyte counts. Vaso-occlusive events are less frequent and less severe in Kuwaiti patients, which may be due to the higher levels of HbF seen in this group. Most of the Kuwaiti patients with Hb SS and Hb Sb-thalassemia have HbF levels between 10-20% into adolescence. In contrast, American cases of Hb SS and Sβ-thalassemia rarely display HbF levels > 10% after ten years of age. (71)

However, sickle cell disease in Kuwait is not "benign." Several Kuwaiti patients with low levels of HbF (2-6%), had frequent painful crises, requiring hospitalization and frequent blood transfusions. Also, longitudinal studies on a larger population of sickle cell patients in Kuwait may reveal complications which may only be apparent in older patients. (71)

VIII. MECHANISMS MODULATING FETAL HEMOGLOBIN LEVELS

Studies lend support to the hypothesis that at least three variables contribute to high HbF levels:

(1) The percentage of RBCs and reticulocytes containing HBF.

(2) The quantity of HbF synthesized within F-cells.

(3) The extent to which preferential F-cell survival increases HbF levels. (28)

Dover et al., concluded that these variables are independently regulated and that the expression of each variable is distinctly different between individual patients. They also determined that the production of HbF may be as great in the absence of heterocellular HPFH as in its presence. While the investigators agreed that HbF offered RBCs protection against premature loss, the degree to which it does so was felt to be variable from one individual to another. The inherent implication of this is that there exists a factor (or factors) other than HbF levels that modifies F-cell production and survival. (28)

An analysis of in vivo changes in F-cell production supports the hypothesis that increased F-cell production is associated with accelerated erythropoiesis. F-cell production in a child with sickle cell anemia was found to be reduced to a greater degree than non-F-cell production when erythropoiesis is suppressed. This lends support to the idea that F-cell production is regulated separately from non-F-cell production. It is not clear if falling erythropoietin levels and subsequent depressed erythropoiesis are associated with selective depression of F-cell production. (29) The authors conclude that individuals with sickle cell anemia like individuals

with normal hemoglobin, can change their relative F-cell production in response to erythropoietic stress via a process that is under a different system of regulation than non-F-cells. (29)

By examining γ globin gene expression, haplotypes associated with elevated HbF levels and the clinical course of sickle cell patients with high HbF, it is hoped that the mechanisms regulating HbF synthesis will be clarified. In the Saudi Arabian population, a milder form of sickle cell disease is found that is associated with high HbF levels. A similar syndrome can also be seen in Asian-Indians with sickle cell disease. Interestingly, unlike Jamaican or American Blacks with sickle cell disease and high hemoglobin F levels, one usually cannot identify in Saudi or Indian families a parent with slight elevation of HbF - a finding that would be consistent with the Swiss type of heterocellular HPFH. (16)

In Swiss type HPFH, an autosomal dominant inheritance pattern leads to slightly elevated HbF levels usually in the range of 2-3% in heterozygotes. The HbF produced has both Gγ and Aγ subtypes of the γ globin chains. Individuals with Swiss type HPFH and a hemoglobinopathy are often asymptomatic. The molecular defect in Swiss type HPFH is unknown. (16)

F-cell production in sickle cell anemia may be regulated by a genetic locus that is linked to or separate from the β globin gene cluster. Attempts to elucidate mechanisms modifying F-cell production are compounded by the preferential survival of F-cells that varies from individual to individual and thus serves as a poor indicator of actual levels of F-cell production. (24) Dover et al. correlated F-reticulocyte levels in sibling pairs with SS disease and

between parents and their children and found that genes controlling F-cell levels are linked to the β globin gene site and that these genes also control F-cell production in non-anemic individuals. (24)

Boyer et al., in a later study, again looked at F-cell production in an attempt to determine if it is regulated by a site (or sites) separate from the β globin gene cluster. Results revealed a substantial number of sibling pairs with discordant F-reticulocyte levels. The discordance was found to be significant and reproducible. Polymorphic markers were used to ascertain that the discordance appeared in siblings who almost certainly had the same mother and father. (12) Boyer et al., concluded that at least one genetic regulator of F-cell production in sickle cell anemia segregated separately from sickle hemoglobin. A gene separate from the β globin gene cluster may regulate F-cell production by elaborating or modifying a substance that interacts with a receptor or effector site on the β globin gene region. Mutations in the modifying substance or gene site itself could then control levels of F-cell production.

Another hypothesis, supported by the finding that HbF synthesis in vivo is usually completed early in erythroid maturation, is that a gene (or genes) can control HbF levels by influencing the percentage of cells in various stages of development when the F-cell "window" is open. (12)

The finding that 5-azacytidine increases F-reticulocytes from 10% to as much as 52% in sickle cell anemia patients has caused cautious optimism that new therapeutic modalities for sickle cell disease may be on the horizon. The rise in F-reticulocyte levels

from the use of 5-azacytidine has been associated with a demethylation of DNA associated with γ globin genes from bone marrow erythroid cells. The persistence of fetal hemoglobin in childhood and adulthood may be due to mutations in the γ globin gene associated DNA which inhibit methylation later in life. (62)

IX. EXPERIMENTAL OBJECTIVES

The purpose of our study was to examine the levels of HbF in patients with sickle cell disease in the Kuwaiti population. Furthermore, an analysis of γ globin gene expression in these patients was undertaken in an attempt to facilitate scientific understanding of the mechanisms involved in increasing γ chain synthesis.

X. METHODS AND MATERIALS

Solutions & Buffers

Solution A: 10% Ammonium Persulfate (fresh)

Solution B: 60% Acrylamide + 0.4% Bis (stored at 4° C; redissolved in a 37°C water bath)

Solution C: 10% Triton X-100

Solution D: (Loading Buffer): 6M Urea + 8% HAC + 8% β-mercaptoethanol + 0.3 mg/ml pyronine Y

1 M Cysteamine

Lysis Buffer: 5mM $MgCl_2$ (stored at 4°C)

Coomassie Blue Stain Solution: .62 g Coomassie Blue in 10% acetic acid and 45% methanol solution

Destain Solution: 10% acetic acid and 10% methanol solution

Acid Acetone: 2 ml 12N HCl + 38 ml acetone + 300l b-mercaptoethanol

Hemoglobin Purification

Blood samples from seven pedigree families and eight individuals with various forms of sickle cell and/or thalassemia

syndrome (including SF, Sickle β-thalassemia, β-thalassemia intermedia) were collected via venipuncture at Al-Sabah Children's Hospital in Kuwait. HbF levels were determined by alkali denaturation.

Erythrocytes from peripheral blood of patients was washed three times in an equal volume of phosphate buffered saline (PBS). Between each washing the sample was centrifuged at 2000 rpm for five minutes in a RC-2 Sorvall refrigerated centrifuge. The serum layer was carefully removed with a Pasteur pipette. The sample was then solubilized at 4° C for 10 minutes in four volumes of lysis buffer. The sample was then centrifuged at 10,000 rpm (using a HB-4 rotor) for 20 minutes. The liquid layer containing hemoglobin was removed with a Pasteur pipette (without disturbing the layer of cellular debris at the bottom of the tube), placed in labelled tubes and stored at -70° C.

Some of our blood samples had lysed prematurely during transit from Kuwait. From these samples globin preps were made using acid acetone. Acid acetone at 4° C was added in a 1:20 volume to our sample. The mixture was centrifuged in a RC-2 Sorvall centrifuge for five minutes at 10,000 rpm. The liquid supernatent layer was discarded and the pellet was washed three times with plain acetone at 4° C. The pellet was then dried using nitrogen gas. The pellet was resuspended in 500 microliters of loading buffer (Solution D), placed in an appropriately labelled tube and stored at -70° C.

Triton Gel Electrophoresis

A 12.5% polyacryalmide gel with 6M urea, 2% Triton X-100 in 5% acetic acid was used to separate the globin chains, using a procedure previously described by Rovera et al. (84)

The gel was prepared by combining 2 ml of solution B + O.5 ml glacial acetic acid + 250λ TEMED (N,N,N',N', - tetramethylenediamine) + 18 grams urea and distilled water to bring the volume up to 49 ml. The solution was vortexed and degassed under a vacuum. Three hundred microliters of solution A and 1 ml of 100% Triton were added to the gel solution. The gel was then poured between two glass plates with spacers. It was overlaid with distilled water and allowed to polymerize. After polymerization occurred the top spacer was removed and the gel was overlaid with distilled water.

The gel was pre-electrophoresed for one hour at 200 V with 5% acetic acid running buffer. The buffer was then removed and the gel was overlaid with 1M cysteamine (10 microliters per well). The reservoir was refilled with 5% acetic acid running buffer and electrophoresed for 45 minutes at 140 V. Buffer was again removed and hemoglobin samples applied (2-5 microliters lysate in 30 microliters of solution D). Normal adult and fetal hemoglobin samples were run as controls. The gel was electrophoresed at 60 volts for 8-16 hours with the current running backwards towards the anode. Gels were stained for 30 minutes with Coomassie Blue and destained by diffusion in 10% acetic acid and 10% methanol. The Gγ/Aγ globin ratio was determined via visual inspection.

XI. RESULTS

Family #	Identification	Diagnosis	%HbF	Gγ/Aγ
Unrelated individuals with high HbF	R.W. (patient)	SF	16%	1:1
	S.A. (patient)	Sickle-β^o thal	15.5%	1:1
	S.D. (patient)	Sickle-β^o thal	21.7%	1:1
	A.S. (patient)	β-thal intermedia	37.5%	1:1
	M.A. (patient)	Sickle-β thal	10.4%	1:1
	H.H. (patient)	SF	16%	1:1
Family #1	Faiza (patient)	homozygous Sβ-thal	60.7%	1:1
	Father	Sickle-β^o thal	9.7%	1:1
Family #2	M.B. (patient)	SF	33%	1:1
	N.J. (mother)	AS	<2%	1:1
	B.O. (father)	AS	<2%	1:1
Family #3	A.S. (patient)	SF	22%	1:1
	S.A. (father)	AS	<2%	1:1
	G.B. (mother)	AS	<2%	1:1
Family #4	W.M. (patient)	ASF	15%	1:1
	H.M. (patient)	SF	- - - *	1:1
	K.M. (mother)	AS	<2%	1:1
	M.T. (father)	AS	<2%	1:1
Family #5	N.A. (patient)	ASF	24%	1:1
	S.A. (patient)	SF	24%	1:1
	M.A. (patient)	SF	- - - *	1:1
	A.I. (father)	AS	<2%	1:1
	A.H. (mother)	AS	<2%	1:1
Family #6	A.N. (patient)	SF	32%	1:1
	Q.N. (sibling)	AS	20%	1:1
	H.N. (sibling)	AS	<2%	1:1
	W.H. (father)	AS	<2%	1:1
	A.A. (mother)	A?	<2%	1:1

Family #7	L.S. (patient)	SF	23%	1:1
	S.J. (father)	AS	<2%	1:1
	S.M. (mother)	AS	<2%	1:1

*--- Denotes inability to accurately quantify %HbF
ASF - Electrophoresis revealed hemoglobins F, A and S

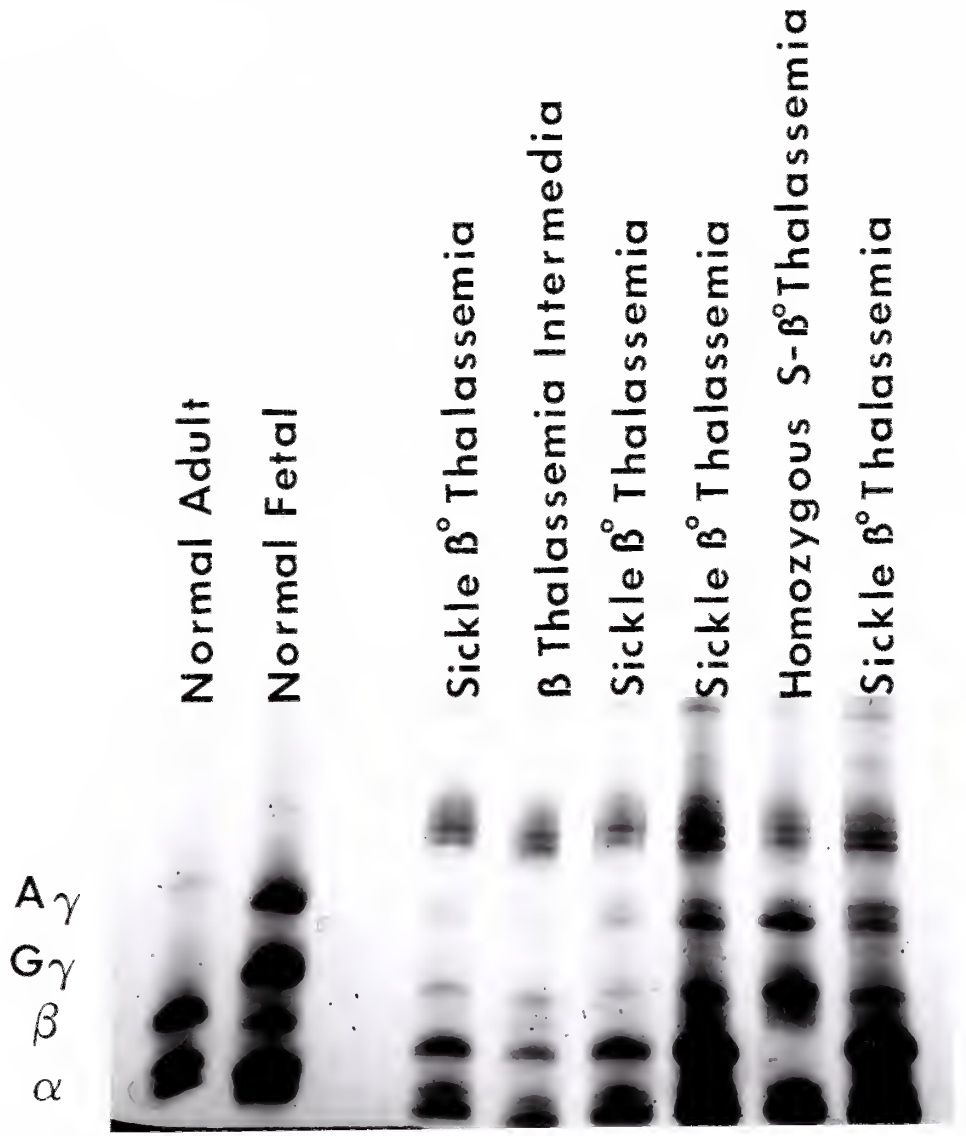
Our data revealed:

(1) All of the Kuwaiti sickle cell disease patients sampled, exhibited high levels of HbF, ranging from 10.4-60.7%

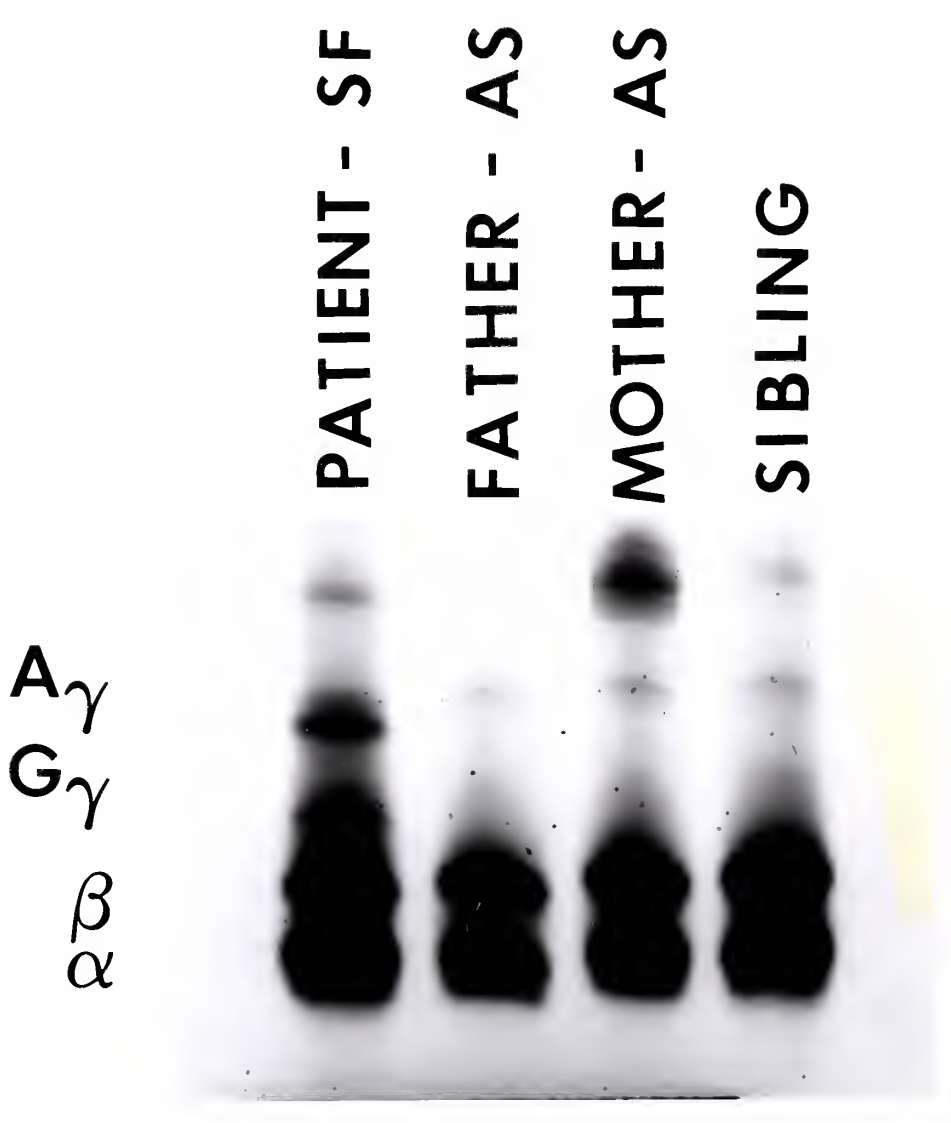
(2) The expression of each of the gamma globin genes, $G\gamma$ and $A\gamma$, is equal in the Kuwaiti patients studied.

(3) Our results were similar to those seen in the analysis of the $G\gamma/A\gamma$ ratio in Saudi Arabians with mild sickle cell disease due to heterocellular HPFH. However, Kuwaitis do not exhibit classical HPFH, as the parents of affected individuals do not have high HbF levels.

RANDOM INDIVIDUALS WITH HIGH FETAL HEMOGLOBIN



FAMILY 7


PATIENT - SF
FATHER - AS
MOTHER - AS
SIBLING
A_γ
G_γ
β
α

mouse model for for sickle cell disease. In a series of experiments, the dominant control region (DCR) sequences which flank the β globin gene locus, were used to direct the copy number dependent expression of human β globin gene in erythroid cells and transgenic mice. By linking the β globin DCR sequences to the human alpha globin gene, high level expression can be obtained. One of the transgenic mice revealed no abnormal somatic development, and no obvious evidence of hemolytic anemia, although HbS concentration was 83%. It is possible that the 17% normal mouse hemoglobin offered some protection against hemolytic anemia, just as similar amounts of fetal hemoglobin appear to protect human β^S/HPFH heterozygotes. Greaves et al. note however that one cannot disregard the physico-chemical properties and microcirculatory changes that may be responsible for this clinical picture. The future development of an animal model for sickle cell disease could serve to further define the factors mediating clinical severity in sickle cell disease as well as screen potential anti-sickling agents. This animal model could also serve as a template for the development of somatic gene therapy using the β globin DCR. (38)

New therapeutic options for the treatment of sickle cell disease may also be developed and the technology of molecular genetics improves. With the advent of recombinant DNA technology, molecular cloning of the human globin gene is now possible. Nucleotide sequences have been modified (by acetylation or methylation of "key" nitrogenous bases) to deliniate elements involved in the regulation of globin gene expression. Use of restriction enzymes has led to the mapping of the various sickle cell

alleles and further understanding of the interaction between sickle hemoglobin states and alpha thalassemia. (10)

The molecular basis for the predominance of γ globin gene expression during gestation and the subsequent switch to β gene expression just prior to birth remains incompletely understood. However, many agree that prevention or reversal of this switch would help to decrease the clinical severity for patients with sickle cell anemia. Such an intervention would decrease or suppress expression of abnormal β genes and replace their gene products with HbF, the product of γ globin gene expression.

Therapeutic manipulation of γ and β globin gene expression may be obtainable via further clarification of the effect of methyl, acetyl or other moieties on particular nitrogenous bases. Hypomethylation has been associated with high levels of gene activity. Disimone et al. in a series of experiments showed that treatment of baboons with the methylation inhibitor 5-azacytidine (a cancer chemotherapy drug) led to high levels of fetal hemoglobin production. The toxicity of 5-azacytidine and unknown effects of the drug on other genes make it unsuitable for widespread clinical use.

The polymerase chain reaction (PCR), designed to enzymatically amplify selective sequences of DNA, allows for sickle cell disease to be identified quickly and easily. PCR and subsequent direct DNA sequencing may allow for identification of deletions, or mutations associated with high HbF levels. While restriction enzyme mapping is a quick and easy technique, it is limited to the linkage analysis of polymorphic restriction sites and to situations

in which the mutation creates or disrupts a restriction site. PCR and subsequent DNA sequencing or analysis with allele specific oligonucleotide probes can serve as a more general means of investigating possible causes of the clinical heterogeneity seen in sickle cell disease. (85)

In summary, we analyzed hemoglobin F levels and expression of the γ globin genes in a population of Kuwaiti patients with mild sickle cell disease. While the Gγ/Aγ ratio of 1:1 was lower than the fetal ratio of 7:3, it remains higher the the 2:3 ration seen in normal adult RBCs. Previous studies have defined an association between high HbF levels and decreased morbidity and mortality in sickle cell patients. We propose that future studies should provide for the cloning and sequencing of the γ and β globin gene region and their promoters as well as in-depth linkage analysis to delineate molecular mechanisms that may serve to increase HbF levels under conditions of severe erythropoietic stress. Molecular determinants of HbF production and/or clinical heterogeneity may be closely linked to the β globin gene cluster, linked to the β globin gene cluster but outside of the region spanning the ε to φβ1- globin genes or farther away on chromosome 11p or even on a different chromosome. (61) It is likely that such linkage analysis will serve to clarify and accurately define on a molecular basis, a number of "sickle cell syndromes" that currently fall under the general rubric of sickle cell disease.

MAJOR SICKLE β GLOBIN GENE HAPLOTYPES

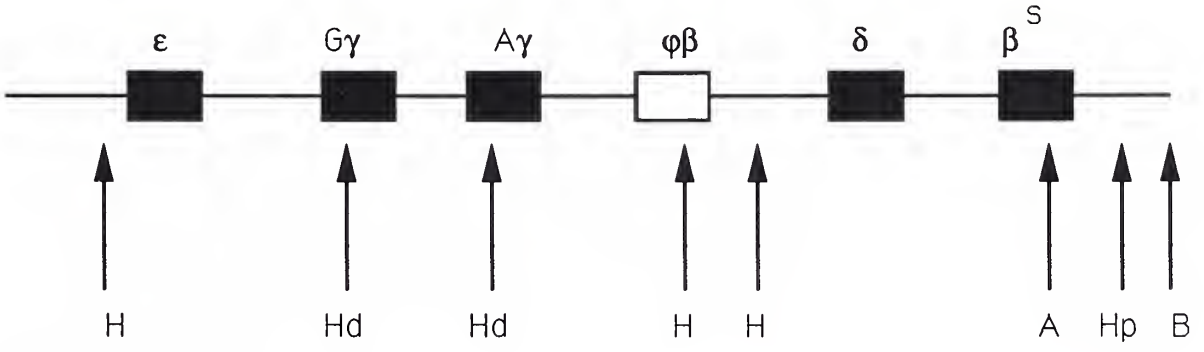


Figure 1
The haplotype is characterized by the presence or absence (+ or -) of an array of of polymorphic restriction sites within the β globin gene cluster. (51)
H = HindII
Hd = HindIII
A = AvaII
Hp = HpaI
B = BamHI

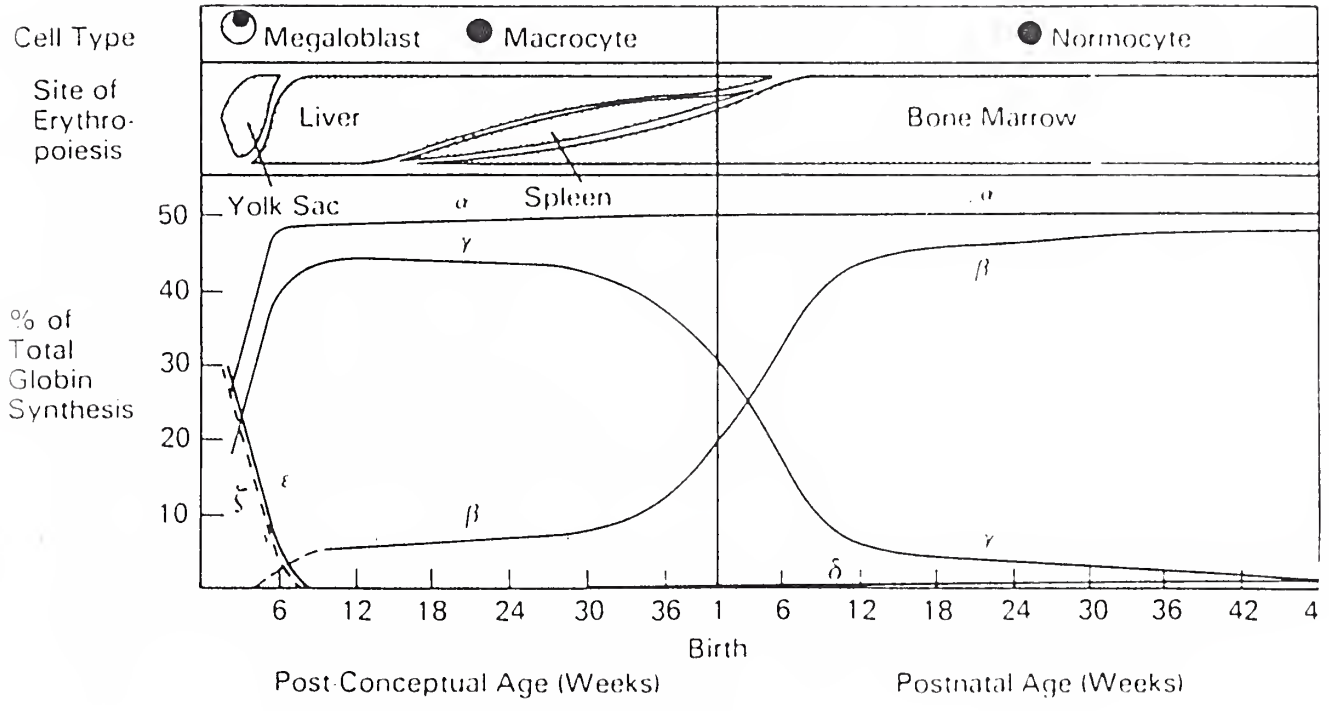


Figure 2: Chart demonstrates changes in globin chain production, sites of hematopoeisis, red cell morphology, and size of erythrocytes during prenatal and postnatal life. (From H. Franklin Bunn and Bernard G. Forget Hemoglobin: Molecular, Genetic and Clinical Aspects. Philadelphia: WB Saunders, 1986)

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