Sickle cell anaemia : comparative clinical and molecular studies of Nigerian and Kuwaiti patients

Citation for published version (APA):

Adekile, A. D. (1996). *Sickle cell anaemia : comparative clinical and molecular studies of Nigerian and Kuwaiti patients*. [Doctoral Thesis, Maastricht University]. Rijksuniversiteit Limburg.
<https://doi.org/10.26481/dis.19960523aa>

Document status and date:

Published: 01/01/1996

DOI:

[10.26481/dis.19960523aa](https://doi.org/10.26481/dis.19960523aa)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
- The final author version and the galley proof are versions of the publication after peer review.
- The final published version features the final layout of the paper including the volume, issue and page numbers.

[Link to publication](#)

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license above, please follow below link for the End User Agreement:

www.umlib.nl/taverne-license

Take down policy

If you believe that this document breaches copyright please contact us at:

repository@maastrichtuniversity.nl

providing details and we will investigate your claim.

Download date: 26 Apr. 2023

**SICKLE CELL ANAEMIA :
COMPARATIVE CLINICAL AND MOLECULAR STUDIES
OF NIGERIAN AND KUWAITI PATIENTS**

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de
Rijksuniversiteit Limburg te Maastricht
op gezag van de Rector Magnificus Prof. mr. M.J. Cohen
volgens het besluit van het College van Dekanen,
in het openbaar te verdedigen
op donderdag 23 mei 1996 om 14.00 uur

door

Adekunle Dada Adekile

geboren te Ibadan, Nigeria, op 27 december 1949

Promotores:

Prof. Dr. J.P.M. Geraedts

Prof. Dr. T.H.J. Huisman (Augusta, Georgia, USA)

Beoordelingscommissie:

Prof. Dr. J.L.H. Evers, voorzitter

Prof. Dr. F.C.S. Ramaekers

Prof. Dr. C.E. Blanco

Prof. Dr. L.F. Bernini (Rijksuniversiteit Leiden)

Prof. Dr. H.K.A. Visser (Erasmus Universiteit Rotterdam)

ACKNOWLEDGEMENTS

I would like to express my deep appreciation to Professor Dr. Joep P.M. Geraedts, University of Limburg, Maastricht, The Netherlands, for his ready support in the realisation of this dissertation. This work could not have been accomplished without the inspiration, guidance, and expert supervision of Professor Dr. Titus H.J. Huisman, Medical College of Georgia, Augusta, GA, USA, to whom I owe enormous gratitude. I thank all my colleagues in Nigeria, the United States, and Kuwait, who contributed in various ways to the work presented in this dissertation. In particular I want to thank Dr. Olugbenga O. Adeodu, Department of Paediatrics, Obafemi Awolowo University, Ile-Ife, Nigeria and Dr. Mohammad Z. Haider of the Department of Paediatrics, Kuwait University. I thank the Chairman, Department of Paediatrics, Kuwait University, Professor Allie Moosa, for his encouragement. The excellent secretarial assistance and moral support of Mrs. Marianne F.H. Carver is most appreciated.

Some of the work presented was supported, in part, by a Fogarty International Research Fellowship IF05TW04415-01, the United States Public Health Service Research Grants HLB-05168 and HLB-41544 (to T.H.J.H.), and Kuwait University Research Grant No. MK031.

DEDICATION

To: Dupe, Ayoola, Debo, Segun, and Akinyinka

CONTENTS

	Abbreviations	i
Chapter 1a	Introduction	3
Chapter 1b	Sickle Cell Anaemia: Review of the Literature	13
Chapter 2	Materials and Methods	41
Chapter 3	Studies Among Nigerians	53
Chapter 3a	Persistent Gross Splenomegaly in Nigerian Patients With Sickle Cell Anaemia: Relationship to Malaria	
	and	
	Clinical and Laboratory Features Associated With Persistent Gross Splenomegaly in Nigerian Children With Sickle Cell Anaemia	55
Chapter 3b	Spleen in Sickle Cell Anaemia: Comparative Studies of Nigerian and U.S. Patients	65
Chapter 3c	Frequency of the α -Thalassaemia-2 Gene Among Nigerian SS Patients and Its Influence on Malaria Antibody Titres	77
Chapter 3d	Haplotypes in SS Patients From Nigeria; Characterisation of One Atypical β^S Haplotype No. 19 (Benin) Associated With Elevated Hb F and High G_γ Levels	85
Chapter 4	Studies Among Kuwaitis	97
Chapter 4a	Influence of α -Thalassaemia Trait on Spleen Function in Sickle Cell Anaemia Patients With High Hb F	99
Chapter 4b	Morbidity, β^S Haplotype, and α -Globin Gene Patterns Among SS Patients in Kuwait	109
Chapter 4c	Molecular Characterisation of α -Thalassaemia Determinants, β -Thalassaemia Alleles, and β^S Haplotypes Among Kuwaiti Arabs	117
Chapter 5	General Discussion	129
Chapter 6	Summary	141
	Samenvatting	147
	Curriculum Vitae	153

ABBREVIATIONS

AAV	adeno-associated virus
ANOVA	analysis of variance
AS	sickle cell trait
ASO	allele-specific oligonucleotide
BSA	bovine serum albumin
CBC	complete blood count
CD(s)	codon(s)
CO-Hb	carbonmonoxy-Hb
cpm	counts per minute
dATP	deoxy adenosine triphosphate
dCTP	deoxy cytosine triphosphate
dNTP	deoxy nucleotide triphosphate
ddATP	dideoxy adenosine triphosphate
DDAVP	desamine-D-arginine vasopressin
ddCTP	dideoxy cytosine triphosphate
ddGTP	dideoxy guanosine triphosphate
ddTTP	dideoxy thymidine triphosphate
DTT	dithiothreitol
dUTP	deoxy uracyl triphosphate
DEAE	diethylaminoethyl-
DNA	deoxyribonucleic acid
DPG	(2,3)-diphosphoglycerate
ECL	enhanced chemiluminescent
EDTA	ethylene diamine tetra acetate disodium
FIGLU	formiminoglutamic acid
Hb(s)	haemoglobin(s)
HPLC	high performance liquid chromatography
HS	hypersensitive site
IEF	isoelectrofocusing
Ig	immunoglobulin
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
ISC(s)	irreversibly sickled cell(s)
IV	intravenous
IVS	intervening sequence
IVS-I	first intervening sequence
IVS-II	second intervening sequence
LCR	locus control region
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCV	mean corpuscular volume
MOPS	(3[n-morpholino]propanesulfonic acid)
mRNA	messenger ribonucleic acid
MW	molecular weight

NS	not significant
nt(s)	nucleotide(s)
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PCV	packed cell volume
PGS	persistent gross splenomegaly
poly A	polyadenylation
PVP	polyvinylpyrrolidone
RBC(s)	red blood cell(s)
RESA	ring-infected erythrocyte surface antigen
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
SCA	sickle cell anaemia
SD	standard deviation
SDS	sodium dodecylsulphate
SE	standard error
SS	sickle cell disease
SSC	sodium saline citrate buffer
SSPE	sodium saline phosphate EDTA buffer
STE	sodium Tris EDTA buffer
TBE	Tris borate EDTA buffer
TE	Tris EDTA buffer
TEA	Tris EDTA acetate buffer
TFA	trifluoroacetic acid
thal	thalassaemia
T _m	melting temperature
TMAC	tetramethyl ammonium chloride
TSS	tropical splenomegaly syndrome
UTR	untranslated region
UV	ultraviolet
vWF	von Willebrandt factor
WBC(s)	white blood cell(s)

CHAPTER 1

Chapter 1a **Introduction**

Chapter 1b **Sickle Cell Anaemia: Review of the Literature**

CHAPTER 1a

INTRODUCTION

Haemoglobinopathies. Haemoglobinopathies are a heterogeneous group of inherited qualitative (abnormal Hbs) or quantitative (thalassemias) Hb disorders. They are the most common monogenic diseases in the world and are responsible for significant morbidity and mortality, especially in developing countries (1,2). The worldwide distribution of the common forms of haemoglobinopathies corresponds to that of current or recent endemic malaria (3-5). There is a variety of epidemiologic and laboratory evidence showing that many of the common mutant alleles, especially in the heterozygote, confer relative protection against the effects of *P. falciparum* malaria (6-8). There has therefore been preferential selection for these traits, thus their current high frequencies in many parts of the world.

Sickle Cell Anaemia (SCA). SCA is the most widely distributed qualitative (or structural) haemoglobinopathy. It reaches its highest frequency (>30%) in parts of tropical Africa, but is also found at polymorphic frequencies in many parts of Europe, the Middle East, the Indian subcontinent, and among the Black communities in the Caribbean, North and South America (3-5).

Recent studies of the genetic epidemiology of SCA have refined our knowledge about the possible origin(s) of the β^S mutation and the several factors that modulate its clinical severity. It is now established that there are several epistatic factors, linked and unlinked to the β -globin gene, that are critical in determining the individual's prognosis (9,10). The major linked factor is the haplotype of the β -globin gene cluster which is determined by the presence of certain polymorphic nt variants that create restriction endonuclease cleavage sites at different locations in the cluster. Each of the common haplotypes tends to be associated with a particular geographic/ethnic zone and the clinical severity of the disease varies accordingly. Unlinked factors include coexistence of α -thal, the patient's gender (females tend to have higher levels of Hb F and a less severe clinical course) and a variety of environmental factors (11,12).

The clinical presentation of SCA is protean and any tissue or organ can be affected. One of the first organs to exhibit pathology is the spleen, so much so that as early as 2 years of age, many of the patients have lost its function (13,14). The consequences of hyposplenism can be quite severe especially in predisposing the patients to serious bacterial infections. However, splenic dysfunction occurs at different rates and to varying degrees in different groups of patients. The factors responsible for this are not quite clear but are probably both genetic and environmental.

Aims of the Dissertation. This dissertation presents some clinical and molecular studies of SCA among patients from two distinct geographic and ethnic backgrounds:

Nigerians (West African Negroes) and Kuwaitis (Middle East Arabs). The aims of the studies are:

A. Clinical:

- 1) To compare the patterns of morbidity from a review of hospital and patients' records;
- 2) to compare the frequencies of splenomegaly and to present the results of splenic function tests (using laboratory and/or radio-scintigraphic studies);
- 3) to identify clinical, molecular, and environmental factors which influence or are related to spleen function in both groups of patients.

B. Molecular:

- 1) To identify prevalent β^S haplotype(s);
- 2) to determine the frequency and types of co-existent α -thal;
- 3) to highlight the differences in the two populations of patients, and their effects on the clinical course of the disease.

GEOGRAPHY OF NIGERIA

Nigeria, the most populous African country with about 100 million people, is located on the West Coast of the continent and shares borders with Niger in the north, Chad and Cameroon in the east, the Gulf of Guinea in the south, and Benin in the west. Nigeria has an area of 923,768 km² and can be divided into four distinct geographical regions (Fig. 1a/1). Along the coast is a belt of mangrove forests and swamps, stretching about 16 km inland in most places. In the Niger delta region, the coastal belt extends about 100 km inland. Beyond the coast is a broad, hilly, forest belt, that gradually rises to the rocky terrain of the Jos and Bauchi plateaus. Beyond these plateaus is a region of savanna that stretches to a semi-desert zone in the extreme north (15) (Fig. 1a/2).

With more than 250 ethnic groups, Nigeria is a complex linguistic, social, and cultural mosaic. More than half the population consists of the Hausa and Fulani peoples of the north, the Yoruba of the southwest, and the Ibo of the southeast. Other ethnic groups include the Edo, Ijaw, and Ibibio of the south, the Nupe and Tiv of the central part of the country, and the Kanuri of the northeast. At least 45% of Nigerians are Muslims, the bulk of whom live in the Hausa, Fulani, and Kanuri areas in the north. Some 38% are Christians, while traditional religions are practiced by the remainder of the population.

Early History. The people of Nigeria are derived from Negro Sudanese/Guinean stock; however, there are a few people of probable Saharan extraction. Legend has it that some early settlers might have originally migrated from the Nile Valley (16,17).

MALI

NIGER

CHAD

KEY

- International Boundary
- ⊙ Abuja—Capital of Nigeria
- State Capitals
- ⊗ International Airports
- ✕ Inland Airports

FIG. 1a/1. Map of Nigeria showing the different states and major cities.

FIG. 1a/2. Map of Nigeria showing the vegetation pattern.

Little is known about the history of Nigeria in ancient times, but archaeologists have discovered evidence of a Neolithic (circa 800 B.C.-A.D. 200) culture at Nok, southwest of the city of Jos in Central Nigeria. The northern part of the present territory of Nigeria was the site of organised states during the Middle Ages. By the 8th century, the region southwest of Lake Chad was part of the Kanem-Bornu Empire, which in 1086 adopted Islam. By about 1300 Bornu was a flourishing centre of Islamic culture, rivaling Mali in the west. Bornu reached its zenith as an independent kingdom under Idris Alooma, who extended his rule over many of the eastern Hausa states that had existed in the area west of Kanem-Bornu since the 11th century. The western states were under the control of the Songhai Empire. Following the break-up of Songhai and the decline of Kanem-Bornu in the late 16th century, the Hausa states regained their independence and continued to flourish until the early 19th century. The Fulani, who then became prominent under Usman dan Fodio, had been established throughout Hausaland since the late 16th century. In the southern part of the country, the Yoruba had their own states in the west, centering on Ife and Oyo; the Edo ruled in Benin in the present south-central parts, and the Ibo in the east and north of the Niger Delta. All these people had functioning states before or around A.D. 1400.

The Portuguese, British, and others established slave-trading stations in the Niger Delta area in the 17th and 18th centuries. The interior was first penetrated by explorers seeking the source of the Niger River, notably the Scottish traveler Mungo Park in 1795 and 1796, and the British explorers Richard and John Lander in 1830 and 1831. In the 19th century, palm oil became so important an article of commerce that the delta region was known as Oil Rivers. Petroleum was discovered in the first half of this century and its export is the mainstay of the national economy.

Frequency of Sickle Cell Trait in Nigeria. Most of Nigeria is endemic for malaria. In the coastal and rain forest areas the infection is holendemic, *i.e.* transmission is continuous and unvaried throughout the year, while in the savanna and semi-desert areas, it is hyperendemic, *i.e.* transmission is continuous, but low during the dry season. The maximum advantage conferred by sickle cell trait in malaria resistance is more pronounced in hyperendemic regions. While the frequency of the sickle cell trait in Nigeria varies from about 25-30%, the highest rates are found in the savanna regions in the north and northeast (18-20).

GEOGRAPHY OF KUWAIT

Kuwait, located in the northeastern corner of the Arabian Peninsula, shares borders in the north and northwest with Iraq, on the east with the Persian Gulf, and in the south with Saudi Arabia (Fig. 1a/3). The country's total area, including the islands of Bubiyan and Failaka, is 17,818 km². Virtually the entire country, desert, with a flat-to-rolling terrain. The average annual temperature is 25°C (79°F), and the average annual rainfall is 127 mm or less, most of which falls in the cooler season between October and March. During the dry season temperatures frequently exceed 46.1°C (115°F). The country obtains its water supply from the desalination of sea-water. Petroleum and natural gas are Kuwait's only natural resources (21,22).

FIG. 1a/3. Map of Kuwait showing the general layout and important landmarks.

The native people are Arabs. However, many minority groups are present, including semi-nomadic Bedouins, Arabs from other countries, Indians, Pakistanis, and Iranians. The population was 1,698,077 in 1993 with an overall density of about 95 persons per square km (23). The city of Kuwait is the seat of government and chief port. Islam is the predominant religion, the large majority being Sunni Muslims. The official language is Arabic, but English is widely spoken. Kuwait is one of the world's richest countries in terms of yearly gross national product per capita.

Early History. The emirate developed around the city of Kuwait, which was settled in the 18th century by migrants from the Najd Province of Saudi Arabia, although the early settlers included people of Iraqi and Iranian ancestry (24,25). Kuwait was nominally under Ottoman Turkish rule until 1899, when the reigning emir asked for, and obtained, British protection. In 1914 Great Britain reaffirmed its protective role and formally recognised the independence of the state. Subsequently, Wahhabis from the Saudi Arabian province of Najd attacked Kuwait. The British aided the emirate, and peace was restored in 1921 by a treaty establishing the Kuwait-Najd border; a neutral zone was created in 1922. Petroleum was discovered in Kuwait in 1938. In 1990, Kuwait was invaded by Iraq and it took the combined effort of the Allied Forces led by the United States of America to dislodge the invaders.

Frequency of Sickle Cell Trait in Kuwait. Although Kuwait is not endemic for malaria, there are areas of endemicity in and around the Middle East. Indeed, the first part of the world where malaria became a public health problem was in the Sumerian civilisation (in present day Iraq) in the Tigris and Euphrates valleys in the early neolithic period. Within the Arabian Peninsula, various oases (mainly in Saudi Arabia) where active agriculture is pursued, are endemic for malaria. Estimates from Oman, the United Arab Emirate, Yemen, and Kuwait have established the frequency of sickle cell trait at about 3% (26,27); however, in areas close to the oases of Saudi Arabia, the figure is as high as 30% (28).

REFERENCES

1. Huisman, T.H.J.: The structure and function of normal and abnormal haemoglobins. In: *The Haemoglobinopathies*, edited by D.J. Weatherall and D.R. Higgs, Bailliere's Clinical Haematology, Vol. 6, page 1, W.B. Saunders Company, London, 1993.
2. Dimovski, A.J.: Factors affecting the fetal hemoglobin levels in patients with sickle cell anemia and thalassemia. Doctoral Thesis, University of Limburg, Maastricht, The Netherlands, 1993.
3. Nagel, R.L. and Ranney, H.M.: Genetic epidemiology of structural mutations of the β globin gene. *Semin. Hematol.*, 27:342, 1990.
4. Nagel, R.L. and Fleming, A.F.: Genetic epidemiology of the β^S gene. In: *Epidemiology of Haematological Disease, Part II*, edited by A.F. Fleming, Bailliere's Clinical Haematology, Vol. 5, page 331, W.B. Saunders Company, London, 1992.
5. Adekile, A.D.: Anthropology of the β^S gene-flow from West Africa to North Africa, the Mediterranean, and Southern Europe. *Hemoglobin*, 16:105, 1992.

6. Allison, A.C.: Protection afforded by sickle cell trait against subtertian malaria infection. *Br. Med. J.*, i:290, 1954.
7. Fleming, A.F., Akintunde, A., Attai, E.D.E., Bradley-Moore, A.M., Greenwood, B.M., Bradley, A., Kirkwood, B.R., and Gilles, H.M.: Malaria and haemoglobin genotype in young northern Nigerian children. *Ann. Trop. Med. Parasitol.*, 78: 395, 1985.
8. Hill, A.V.S.: Molecular epidemiology of the thalassaemias (including Haemoglobin E). In: *Epidemiology of Haematological Disease, Part II*, edited by A.F. Fleming, Bailliere's Clinical Haematology, Vol. 5, page 209, W.B. Saunders Company, London, 1992.
9. Powars, D.R.: Sickle cell anemia: β^S -gene cluster haplotypes as prognostic indicators of vital organ damage. *Semin. Hematol.*, 28:202, 1991.
10. Nagel, R.L.: Severity, pathobiology, epistatic effect and genetic markers in sickle cell anemia. *Semin. Hematol.*, 28:108, 1991.
11. Embury, S.H.: The interaction of α -thalassemia with sickle cell anemia. *Hemoglobin*, 12:509, 1988.
12. Steinberg, M.H., Hsu, H., Nagel, R.L., Milner, P.F., Adams, J.G., Benjamin, L., Fryd, S., Gillette, P., Gilman, J., Josifovska, O., Hellman-Erlingsson, S., Safaya, S., Huey, L., and Rieder, R.F.: Gender and haplotype effects upon hematological manifestations of adult sickle cell anemia. *Am. J. Hematol.*, 48:175, 1995.
13. Pearson, H.A., Spencer, R.P., and Cornelius, E.A.: Functional asplenia in sickle cell anemia. *N. Engl. J. Med.*, 281:923, 1969.
14. Adekile, A.D., McKie, K.M., Adeodu, O.O., Sulzer, A.J., McKie, V.C., Kutlar, F., Ramachandran, M., Kaine, W., Akenzua, G.I., Akolo, A.A., Asindi, A.A., Obinyan, E.A., Ogala, W.N., Ibrahim, M., and Huisman, T.H.J.: The spleen in sickle cell anemia: Comparative studies of Nigerian and U.S. patients. *Am. J. Hematol.*, 42:316, 1993.
15. "Nigeria" Microsoft (R) Encarta. Copyright (c) 1994 Microsoft Corporation. Copyright (c) 1994 Funk & Wagnall's Corporation.
16. Davidson, B.: *African Civilization Revisited From Antiquity to Modern Times*, Citadel Press, Carol Publishing Group, New York, N.Y., 1990.
17. Oliver, R. and Fage, J.D.: *A Short History of Africa*, Penguin Books, London, England, 1988.
18. Livingstone, F.B.: *Frequencies of Hemoglobin Variants*, Oxford University Press, New York, N.Y., 1985.
19. Fleming, A.F., Storey, J., Molineaux, L., Kagan, I., and Cornille-Brögger, R.: Abnormal haemoglobins in the Sudan savanna of Nigeria. I. Prevalence of haemoglobins and relationships between sickle cell trait, malaria and survival. *Ann. Trop. Med. Parasitol.*, 73:161, 1979.
20. Roberts, D.F., Lehmann, H., and Boyo, A.E.: Abnormal haemoglobins in Bornu. *Am. J. Phys. Anthropol.*, 18:5, 1960.
21. "Kuwait" Microsoft (R) Encarta. Copyright (c) 1994 Microsoft Corporation. Copyright (c) 1994 Funk & Wagnall's Corporation.
22. Kuwait University. Welcome to Kuwait and Kuwait University. Handbook, Kuwait University Press, Kuwait, 1992.
23. Central Statistics Office. Annual Statistical Report, Ministry of Planning, Kuwait Statistical Abstract Series, 1993.

24. Abu-Hakima, A.M.: *The Modern History of Kuwait*, page 1, Luzac & Co. Ltd., London, 1983.
25. Abu-Hakima, A.M.: *History of Eastern Arabia: The Rise and Development of Bahrain, Kuwait and Wahhabi Saudi Arabia*, Probsthain, London, 1988.
26. White, J.M., Byrne, M., Richards, R., Buchanan, T., Katsoulis, E., and Weerasinghi, K.: Red cell genetic abnormalities in Peninsular Arabs: Sick cell haemoglobin, G6PD deficiency and α and β thalassaemia. *J. Med. Genet.*, 23:245, 1986.
27. Ghosh, K., Shaqalaih, A., Salman, A., and Hassanein, A.A.: Haemoglobinopathies in a large hospital in Kuwait. *Haematologia*, 25:185, 1993.
28. El-Hazmi, M.A.F.: Haemoglobin disorders: A pattern for thalassaemia and haemoglobinopathies in Arabia. *Acta Haematol.*, 68:43, 1982.

CHAPTER 1b

SICKLE CELL ANAEMIA: REVIEW OF THE LITERATURE

Historical Aspects. Sickle cell anaemia (SCA) or Hb SS disease was first described in the Western literature in 1910 when Herrick reported "peculiar, elongated and sickled" erythrocytes in the peripheral blood smear of an anaemic West Indian immigrant (1). However, there is linguistic and other historical evidence to show that West Africans had recognized the disease for many generations. Africanus Horton (1874) is credited with the first recorded description of the disease in Africa, documenting the fever, crises, joint pains, and constant "abnormality" of the blood as characteristic of the disease (2). The discovery of the sickling phenomenon in the father of the third reported case of SCA led Emmel to suggest a genetic basis for the disease in 1917 (3). The next major advance was in 1949 when Pauling et al (4) demonstrated a difference in electrophoretic mobility between the Hb from an SS patient and a normal individual, while a sickle cell trait (AS) individual had both types of Hb in almost equal amounts. In 1956 Ingram (5) showed that the basic molecular aberration in Hb S was the substitution of valine for glutamic acid in the sixth position of the β -globin chain. In the last two decades, advances in molecular biology, especially gene mapping on the basis of RFLP and PCR, have led to accelerated accumulation of new knowledge about SCA and related haemoglobinopathies.

Pathophysiology. The major pathophysiological consequence of the molecular aberration in the Hb S molecule is its decreased solubility, especially upon deoxygenation. The solubility of deoxy Hb S is only about 1% that of oxy Hb S, while it is only 10% of deoxy Hb A (6). In high concentrations, a solid gel is formed by polymerisation of the deoxy Hb S molecule that alters the shape of the RBC and is responsible for its shortened life-span and rigidity of its membrane (7). Intravascular sickling is responsible for the recurrent vascular occlusion that is so characteristic of the disease. However, there are several other factors that affect the rate of sickling and/or the adhesion of the sickled cell to the venular endothelium (8-10). These include the intracellular Hb F and 2,3-DPG levels, ionic transfers that take place across the RBC membrane (leakage of potassium, with an influx of sodium and calcium), the von Willebrandt factor (vWF), low oxygen tension, decreased pH, increased temperature, advanced cell age, and increased intracellular Hb S concentration (8-15). *In vivo* observations have shown that RBCs in SCA are capable of repeated cycles of sickling and unsickling depending on whether they are in the venous or arterial circulation. However, after several cycles, some RBCs lose the capacity to return to the normal shape even after exposure to oxygen. These are the ISCs seen in peripheral blood smears. Some relationship has been demonstrated between ISC counts and some of the features and complications of the disease (16).

Sickle Cell Disease Spectrum. The term sickle cell disease refers to a condition in which an individual has inherited two abnormal β -globin genes, at least one of which is β^S , and the resulting symptomatology or pathology is attributable to the

sickling phenomenon. The most common and most severe is sickle cell disease, also called sickle cell anaemia, while other common related disorders include Hb SC disease, Hb S- β^0 -thal, and Hb S- β^+ -thal. Other abnormal Hbs, e.g. Hbs D-Punjab, O-Arab, E, etc., may co-polymerize with Hb S to produce sickle cell disease, however, they are rare clinical entities (17,18). It should be noted that sickle cell trait (AS), in which the individual has a normal β^A in addition to the β^S gene, with Hb S concentration usually between 30 and 40%, is not considered as part of the sickle cell disease spectrum. These individuals are essentially normal and do not show signs of disease except in extraordinary circumstances.

Inheritance. The inheritance of sickle cell disease follows simple Mendelian principles. Therefore, when one parent is heterozygous for the β^S gene (AS) and the other parent is normal (AA), the offspring has an equal chance of having either the sickle cell trait (AS) or a normal AA genotype. If both parents are trait individuals (AS), there is a 50% chance of the offspring having the sickle cell trait (AS), and a 25% chance of having a normal AA genotype, and also a 25% chance of having sickle cell anaemia (SS). This probability remains true for each pregnancy regardless of the result of previous pregnancies.

Geographical Distribution of Hb S. Although Hb S is most common in people of African ancestry, it is not limited to Blacks. While it is found at high frequencies in most parts of Africa, it is also found at lower frequencies in the Mediterranean countries of Southern Europe (especially Greece, Italy, Portugal, Turkey, Albania), Eastern Europe [Former Republic of Yugoslavia, Republics of the Former Soviet Union (Georgia, Azerbaijan, Kazakhstan)], the Middle East (Arabian Peninsula, Iraq, Iran, Afghanistan), and the Indian subcontinent (19-21). There is ample evidence to show that the sickle cell trait confers appreciable protection against the complications of *Plasmodium falciparum* malaria (22-24). This has led to preferential selection of the β^S gene and its persistence as an example of balanced polymorphism. It is interesting that the geographical distribution of sickle cell disease corresponds to areas of the world where malaria is endemic either currently or until recently.

Haplotypes and the Origin(s) of the β^S Gene. The genes controlling the synthesis of the non- α -globin chains are located on the short arm of chromosome #11 and are arranged as follows: 5'- ϵ - γ - δ - $\psi\beta$ -3'. Transcription of these genes follows a developmental, stage-specific fashion referred to as Hb switching (25-27). The ϵ chains are only synthesised in the embryonic period, while γ chains are found mainly in the fetus (Hb F); after birth the levels decrease rapidly until about the age of 6 months when the adult levels of less than 1% are found in normal individuals. The δ and β chains are the predominant adult types and are synthesised mainly in postnatal life (18).

Using gene mapping techniques (restriction endonuclease digestion of genomic DNA, electrophoretic separation of the DNA fragments, and identification of the structural genes in these fragments by hybridisation analyses) several polymorphisms or variants within the β -globin gene cluster have been described (19,28-30). The presence or absence of these restriction enzyme sites (Fig. 1b/1) on a particular chromosome is determined by its DNA nucleotide sequence and they create a pattern referred

Haplotype	1	2	3	4	5	6	7	8	9
Saudi Arabian/Indian (#31)	+	+	+	-	+	+	+	+	-
Senegal (#3)	-	+	+	-	+	+	+	+	+
Benin (#19)	-	-	-	-	-	+	+	-	+
Bantu (#20)	-	-	+	-	-	-	+	+	+
Cameroon (#17)	-	-	+	+	-	+	+		+

FIG. 1b/1. Restriction sites in the β -globin gene cluster used for the detection of haplotypes of a chromosome. The five most common β^S haplotypes are also given.

to as the haplotype of the chromosome. ASO probes corresponding to the sequences across these polymorphic sites are easily synthesised. It is now possible to use these to screen large numbers of chromosomes by hybridisation techniques. This has proven to be a far faster and more reliable method of haplotyping and is becoming the more frequently used technique (31-33).

Certain β^S haplotypes segregate in well-defined geographical areas and ethnic groups. Application of this knowledge to anthropological studies has led to the deduction that the β^S gene probably originated by multiple independent mutations on chromosomes of different genetic backgrounds in Africa and once in (Saudi Arabia/India) Asia (34,35). The prevalent β^S haplotype in Atlantic West Africa is referred to as the Senegal or haplotype #3. In Central West Africa and North Africa, it is the Benin or haplotype #19, and in Central and East Africa it is the Bantu or haplotype #20, while the Saudi Arabia/India haplotype is also known as haplotype #31. Haplotype #17 or the Cameroon haplotype is found mainly among the Eton tribe of Cameroon. Fig. 1b/2 shows the distribution of the different β^S haplotypes in Africa. Among Blacks in America and the Caribbean, different haplotypes are encountered depending on which part of Africa their ancestors came from originally. While the predominant haplotype in the western provinces of Saudi Arabia is #19 (Benin), haplotype #31 is the type commonly seen in the eastern provinces, and from there eastward and up to the Indian subcontinent, hence it is referred to as the Saudi Arabia/Indian haplotype (36).

LABORATORY DIAGNOSIS

Haematological Evaluation. Complete blood count (Hb, PCV, RBC, MCV, MCH, MCHC), reticulocyte count, and an analysis of a peripheral blood smear should be the starting point in investigating suspected sickle cell disease. These will indicate the degree and type of anaemia and ISCs may be seen in the smear. There are also tests that indicate the specific presence of Hb S, but do not define the Hb genotype of the individual. These are based either on the morphological changes that occur in RBCs containing Hb S when subjected to deoxygenation or on the poor solubility of Hb S in solutions of high molarity. They are not useful in the newborn period because of the preponderance of Hb F. The classical sickling test uses 2% sodium metabisulphite to rapidly induce deoxygenation when mixed with a drop of blood on a microscope slide, covered with a slip and sealed with wax. The test is read after 24 hours when the typical sickled cells are easily demonstrable under the microscope. Several solutions of high molarity, concentrated phosphate buffers, lysing and reducing agents (*e.g.* sodium dithionite) have been used as screening tests for Hb S. In such solutions, a haemolysate containing Hb S will be cloudy, while those without it remain clear. More accurate is the immunologic procedure that detects Hb S because the monoclonal antibody is directed against the $\beta 6$ Glu→Val replacement.

Hb Electrophoresis and IEF. The conventional method of confirming the diagnosis of SCA is by Hb electrophoresis, usually in an alkaline medium (cellulose acetate, pH 8.4-8.9), in which Hb S migrates towards the anode at a much slower rate than Hb A. The drawback of this system is that it fails to differentiate Hb S from other Hbs

FIG. 1b/2. Distribution of the more common β^S haplotypes among African populations (modified from Ref. 34).

(e.g. Hb D) which have similar electrophoretic mobilities. Electrophoresis on acid agar (pH 6.0-6.2) provides useful confirmatory evidence of the presence of Hb S. In this system, the mobility does not depend solely on electrical charge; Hb F migrates slightly towards the cathode and Hb S and Hb C towards the anode in characteristic positions, whereas most other variants remain in the position of Hb A close to the origin (17).

In IEF, proteins are fractionated according to their isoelectric points along a pH gradient. This is established either by a stack of isoelectric ampholytes sorted by passage of the current or by a charged matrix obtained by co-polymerisation of varying amounts of acidic and basic acryloyl-derivatives within an acrylamide gel (37). Precast agar gels are commercially available and common conditions such as AS, SS, AC, and AG are readily detected, but even more complicated combinations such as that of a β chain variant (Hb S) and an α chain variant are readily recognised. IEF is the diagnostic method of choice in most centres that have newborn screening programmes because it provides a clear demarcation of Hb F from the minor quantities of adult-type Hbs that may be present in the sample (Fig. 1b/3).

HPLC. This type of chromatography has brought a radical change in the identification and quantitation procedures of Hb variants. Three different techniques are commonly used, namely a) cation exchange HPLC for the separation of Hbs; b) reversed phase HPLC for the separation of globin chains, and c) a different type of reversed phase HPLC used for the separation of fragments of the globin chains obtained by proteolytic digestion (38-42). These techniques are very useful in differentiating and characterising the various forms of sickle cell disease (SS, SC, S- β -thal, and other rarer types) and for identifying those that are associated with high Hb F and high G_γ levels (33,43).

Sequence Analysis of Amplified DNA. During the past 10 years direct analysis of DNA from patients with different forms of haemoglobinopathies has become a most powerful tool in delineating the molecular pathology of these various disorders. DNA is isolated from WBCs of some 5-10 ml blood, collected in EDTA. Next, a segment of DNA of the globin gene where the mutation is located is amplified and sequenced (44-47). A modification of this technique makes use of synthetic oligonucleotide probes, mainly to identify known variants. The amplified DNA samples are dot-blotted onto nylon membranes and hybridised with synthetic oligonucleotides (Fig. 1b/4) that are specific for the normal and mutated sequences (33,43). The method can be applied to the minute quantity of DNA that can be obtained from a dried blood spot collected on filter paper. These molecular techniques have been useful in population studies to identify prevalent haplotypes of the β^S -globin gene in various societies (32), and they have been applied to prenatal diagnosis.

Prenatal Diagnosis was originally performed with electrophoretic studies on blood obtained from the placenta or fetus using a fetoscope. However, it is now possible to isolate DNA from amniotic cells or chorionic villi in the first trimester of pregnancy and subject this to gene mapping or hybridisation studies (48-50).

FIG. 1b/3. Separation of various α and β chain variants present in red cell lysates of newborn babies and adults by IEF.

AS	SS	SS	AS	AS	AS
SS	SS	SS	SS	SS	AS
SS	SS	AS	SS	AS	AS
SS	SS	SS	Sβ	Sβ

Mutant

Normal

PROBES: NORMAL: 5' G•ACT•CCT•G A G•GAG•AAG•TCT 3'
 MUTANT: 5' G•ACT•CCT•G T G•GAG•AAG•TCT 3'
 CODON: 4 5 6 7 8 9

FIG. 1b/4. Identification of the AS, SS, and S-β-thal conditions by hybridisation of amplified DNA samples with appropriate ³²P-labelled oligonucleotide probes.

FACTORS AFFECTING CLINICAL SEVERITY

The symptomatology of sickle cell disease is diverse and any organ or system in the body can be affected, depending upon the age of the patient. However, the disease is characteristically very variable in its clinical severity (51). Some patients hardly have any problems, while others have frequent crises and complications. In the individual patient, the severity may also vary from time to time. Some of the factors that influence this severity include:

- a) **Co-existent α -Thal.** α -Thal decreases the rate of haemolysis by decreasing the MCHC, thereby resulting in higher Hb, PCV, and RBC values. Complications such as leg ulcers, renal pathology, and strokes are fewer and overall survival may also be enhanced (51-57).
- b) **Hb F Levels.** Hb F does not co-polymerise with Hb S and SS patients with elevated Hb F levels usually have a milder clinical course (51). This is because a mixture of Hb S and Hb F has a high solubility and it is likely that the formation of an asymmetrical hybrid ($\alpha_2\gamma\beta^S$) is the primary cause of the inhibition of polymerisation of Hb F (18,58).
- c) **RBC Enzyme Deficiencies.** There is evidence that patients with associated enzyme deficiencies, *e.g.* G-6-PD, may be more prone to repeated haemolytic crisis (59).
- d) **Environmental Factors.** These play a significant role in the morbidity of sickle cell disease. Endemic infections (*e.g.* malaria) are known to frequently precipitate crises. Optimal nutritional intake and adequate and prompt medical care are very important.

The possibility of manipulating Hb F levels in SS patients either with pharmacological agents or genetic engineering has stimulated studies of the factors that influence Hb F synthesis. These factors include:

- a) **Age.** As in the normal population, Hb F levels are highest in the immediate postnatal period. However, they subsequently tend to remain far above the percentages observed in normal individuals of comparable age (60).
- b) **Gender.** It has been suggested that a gene linked to the X chromosome and located at Xp22.2 influences the F cell production in both normal and SS patients (61,62); females with two X chromosomes are homozygous and may express higher levels of Hb F. Support for this possible link has been provided by several other investigators (63-66) who have demonstrated higher levels of Hb F in female SS patients, particularly over the age of 40 years. Figs. 1b/5 and 1b/6 illustrate the influence of gender on Hb F levels in some of our patients with different haplotypes.
- c) **α -Thal.** The existence of a severe α chain deficiency decreases the level of Hb F, presumably because the formation of $\alpha\beta^S$ dimers is preferred over that of $\alpha\gamma$ dimers when the α chain pool is decreased (18). Also, the lower levels of Hb F in SS patients with α -thal is due to the preferential survival of non-F cells because of decreased MCHC values and a consequent decrease in the rate of haemolysis.

FIG. 1b/5. The levels of Hb F in young children with sickle cell anemia, aged 1-12 years. A comparison is made between boys and girls with different haplotypes (modified from Ref. 60).

FIG. 1b/6. The levels of Hb F in young children with sickle cell anemia, aged 1-12 years. A comparison is made between patients with distinct β^S haplotypes (modified from Ref. 60).

Mutations in the β -Globin Gene Cluster.

- a) Mutations within the promoter regions of the G_γ - and A_γ -globin genes are directly related to an increased production of G_γ chains and an elevated Hb F level (31,32). The C $\rightarrow$ T substitution at -158 (G_γ) that creates an *Xmn* I enzyme restriction site is almost always associated with increased G_γ chain levels and, quite often, increased Hb F levels (60,67). This substitution is characteristic of β^S haplotypes #3 and #31 (Senegal and Saudi Arabia/India, respectively), and is probably responsible for the mild clinical course in patients with these haplotypes (67,68).
- b) **LCR.** The β -globin gene cluster LCR consists of a series of five DNase I hypersensitive sites (5' HS-1, -2, -3, -4) located 5' to the ϵ -globin gene and 3' to the β -globin gene (3' HS-1). The LCR controls the sequential developmental stage-specific transcription (Hb switch) of the genes in the cluster (26-28). More recent data have indicated that haplotype-specific sequence variations in 5' HS-2 are related to elevated Hb F levels (69,70).
- c) Elion et al (71) studied the (AT) $_X$ (T) $_Y$ polymorphic region -530 5' to the β -globin gene and found specific variations associated with particular haplotypes affecting the binding of the putative repressor nuclear protein BP1. A tighter binding of this protein with the polymorphic pattern was reported in SS patients with chromosomes of haplotype #31 background as well as lower levels of Hb S in AS heterozygotes (71,72). More recent data have, however, cast doubts on this hypothesis (73).

Clinical Features. Most SS patients do not show any sign of the disease in early infancy, mainly because the predominant Hb at this time is Hb F. However, at about the age of 6 months or just before then, most patients will start to show symptoms of the disease. The early signs include pallor and jaundice as a result of chronic haemolysis, and enlargement of the liver and spleen (hepatosplenomegaly) as a reflection of extramedullary haemopoiesis. The earliest sign of vascular occlusion, from intravascular sickling, is the hand and foot syndrome or dactylitis which is a painful, bilateral swelling of the hands and feet (including the digits), resulting from ischaemic inflammation. It tends to be recurrent and is more common in patients below the age of 4 years (74).

Sickle cell disease is characterised by recurrent episodes of acute illness euphemistically referred to as "crises". Two broad types of crisis are recognised: Vaso-occlusive crisis and acute anaemic crisis.

Vaso-occlusive (painful or thrombotic) Crisis. It is caused by occlusion of blood vessels by sickled cells resulting in pain from ischaemic tissue injury. It can affect any part of the body, but it is particularly common over the long bones, abdomen, chest, and the back. Precipitating factors include physical exertion, exposure to extremes of weather, fever, dehydration, and emotional disturbance, although in many instances, no precipitating events can be identified. The pain usually lasts for 4-6 days, but may persist for weeks.

In infancy, vaso-occlusive crisis usually manifests as the hand and foot syndrome, associated with irritability and refusal to walk. In childhood, the pain may be symmetrical, asymmetrical, or migratory. It may or may not be associated with fever and localised swelling, tenderness or warmth. It may be difficult to differentiate from acute bone infection (osteomyelitis), infarction or septic arthritis. When it affects the abdomen, it should be differentiated from an acute surgical problem or a medical or gynaecologic condition, *e.g.* pancreatitis, cholecystitis, urinary tract infection, pelvic inflammatory disease, ovarian torsion, etc. Right upper quadrant syndrome refers to pain in this region of the abdomen and should be differentiated from gallbladder disease or hepatic pathology. Acute chest syndrome refers to pain in the chest which has to be differentiated from pneumonia or pulmonary infarction.

There are no diagnostic laboratory findings in vaso-occlusive crisis; however, baseline CBC should be obtained. Tests to rule out any underlying precipitating factors should be carried out as indicated. Management is directed at treating any underlying problems, especially infections. Hydration is of paramount importance because dehydration, from insensible water loss, reduced fluid intake or polyuria, promotes sickling. Liberal oral fluid intake may be sufficient in mild cases of vaso-occlusion, but parenteral infusion is indicated for moderate-to-severe cases. Analgesics should be given promptly and in adequate doses. Non-narcotic agents, *e.g.* acetaminophen, are used for mild pain. Non-steroidal anti-inflammatory agents are also useful, but narcotic agents may be necessary for severe pain. Sodium bicarbonate and oxygen are indicated if acidosis or hypoxaemia, respectively, exists.

Hyperhaemolytic Crisis. Although the life-span of RBCs in SS patients is considerably shortened by chronic haemolysis, there are situations when there is an exacerbation of this haemolytic process. Bacterial infections, *e.g.* pneumonia, acute osteomyelitis, urinary tract or parasitic infections (*e.g.* malaria), may precipitate increased haemolysis. Patients with RBC enzyme deficiencies, *e.g.* G-6-PD deficiency, are probably more prone to this complication (59). The patients present with increased pallor, jaundice, and hepatosplenomegaly. There is usually reticulocytosis accompanying a fall in PCV and Hb. Treatment of the underlying condition usually restores the Hb to steady state levels. Severe cases may require a blood transfusion.

Aplastic Crisis. This is characterised by an acute failure of erythropoiesis, usually following a vague upper respiratory tract infection. Affected patients present with weakness and increasing pallor, with the Hb and PCV values falling progressively. At the onset, the reticulocytes are low in number or absent. However, the condition is self-limiting and recovery usually takes place in 2-3 weeks. In the recovery phase, the reticulocyte count increases and the Hb and PCV slowly return to normal. There is evidence that aplastic crisis is associated with parvovirus infection (75).

Acute Sequestration Crisis. This is characterised by sudden onset of progressive anaemia, splenic enlargement, and in severe cases, signs of peripheral shock. It is caused by trapping of a significant proportion of the red cell mass within the enlarged spleen. The precipitating factor is unknown and the patient presents with low Hb, low PCV, decreased platelets, and increased reticulocytes. It tends to be recur-

rent. Treatment of severe cases is an immediate blood transfusion. Recurrence may be prevented by regular blood transfusions or splenectomy (76).

Megaloblastic Change. Because of the higher folate requirements associated with chronic haemolysis, SS patients are prone to megaloblastic episodes consequent on folate deficiency. Other factors, *e.g.* dietary deficiency, recurrent infections, malaria, etc., may predispose to folate deficiency. The patients present with increasing anaemia, low Hb and low reticulocyte counts; there is anisocytosis, poikilocytosis, macrocytosis, and large numbers of nucleated RBCs. Neutrophils show hypersegmentation. Serum folate levels are low and there is an increased excretion of its intermediate metabolite, formiminoglutamic acid (FIGLU). Prophylactic daily folic acid supplementation (1-5 mg) is given almost routinely to SS patients at most centres, although there is some controversy about its justification.

MANIFESTATIONS IN DIFFERENT ORGANS AND SYSTEMS

The Immune System. SS patients are particularly prone to bacterial infections; this constitutes the major cause of mortality among children with the disease (77,78). The common causative organisms include *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Salmonella species*, *Mycoplasma*, *Staphylococcus aureus*, *Escherichia coli*, and *Streptococcus pyogenes*.

Splenic hypofunction, defective opsonisation, and abnormal leucocyte phagocytic function are some of the identified defects in the immune system in SS patients (79-82). The other factor that contributes to their predisposition to bacterial infections is the recurrent vaso-occlusion resulting in necrotic tissue that may harbour certain types of organisms. The latter is particularly important in the pathogenesis of *Salmonella osteomyelitis* (83).

The Spleen. The spleen usually becomes palpably enlarged within the first 2 years in the life of an SS patient because of extramedullary haemopoiesis, congestion, and occasionally, sequestration (80). However, the sluggish blood circulation within the spleen encourages recurrent vaso-occlusion and infarction. This eventually leads to splenic fibrosis with regression in size of the organ as the child gets older (79,80,84). Even before the spleen becomes fibrotic, there is evidence that its function is compromised early in the life of the patient. This is referred to as functional hyposplenism and is demonstrable by non-uptake of labelled colloid by the spleen.

The Liver. Hepatomegaly occurs in between 40 and 80% of childhood SS patients (78). Intra-sinusoidal sickling frequently leads to sinusoidal obstruction and dilatation with lymphocytic infiltration. Kupffer cells are usually swollen following erythrophagocytosis. Some children show evidence of focal fibrosis and necrosis. Hepatic enzymes are often elevated in steady state and the values increase with age and during episodes of painful crisis. Marked acute elevations of conjugated bilirubin values are associated with intrahepatic cholestasis, viral hepatitis, sickle cell hepatopathy (or hepatic crisis), and extrahepatic obstruction (85).

The Gallbladder. Chronic haemolytic anaemia and consequent hyperbilirubinaemia predispose SS patients to the development of pigment gallstones (86). It tends to be more common in the older age groups and there is a female preponderance, especially after puberty. There is also a correlation between biliary tract infection and frequency of stones (87). While regular ultrasonographic abdominal scanning for gallstones is recommended, the issue of elective cholecystectomy for all diagnosed cases is still controversial, especially when they are asymptomatic.

The Kidneys. The haemodynamic changes of chronic anaemia and the recurrent vaso-occlusion of sickle cell disease result in diverse renal manifestations. Hyposthenuria (low urinary specific gravity) occurs in all individuals who carry the β^S gene, including sickle cell trait (88). This leads to polyuria, nocturia, and probably contributes to enuresis (bed-wetting in late childhood). Haematuria may result from micro-infarction of the renal pyramids in sickle cell disease and in sickle cell trait. Nephrotic syndrome and chronic renal failure have been reported in older SS patients (89,90).

The CNS. The most severe CNS complication is stroke from cerebrovascular accidents (91,92) which may present with transient features, *e.g.* convulsions, stupor, coma, dysphasia, visual disturbances (93), hearing loss (94), etc. Some cases progress to hemiplegia that results from cerebral infarction in children and intracranial haemorrhage in adults. Hemiplegia is usually of sudden onset, although gradual progression is occasionally seen. It occurs more commonly in children below the age of 14 years. Stroke tends to be recurrent, especially within 3 years following the initial episode. It usually results from partial or complete occlusion of major cerebral vessels. The most common angiographic finding is stenosis or occlusion of one or both internal carotid arteries. Occlusion of the anterior or middle cerebral arteries is the next common finding, while involvement of the vertebro-basilar arteries appears to be rare (92).

The Eyes. Many of the tissues in the eye may be affected in sickle cell disease. Saccular dilatations have been reported in the conjunctival vessels. In the anterior chamber, ischemia leads initially to conjunctival injection, corneal oedema, keratic precipitates, necrotic lens tissue, low intra-ocular pressure, and a dilated, unresponsive pupil. In the posterior chamber, non-proliferative or proliferative retinopathy may occur (95). The latter is more common in Hb SC disease, but it is also seen in SS and S- β^0 -thal.

The Lungs. Quite often patients present with acute chest syndrome that is characterised by chest pain, fever, prostration, and pulmonary infiltrates on chest X-ray (96,97). In children, it usually results from an infectious etiology, while in adults it is more often due to acute pulmonary thrombo-embolism, although bacterial or viral infections could be responsible (98-100). Clinically it is often difficult to differentiate infarction from infection.

The CVS. The only consistent CVS findings in SS patients are caused by chronic anaemia. Thus, cardiomegaly with left and right ventricular hypertrophy occurs in both children and adults. Myocardial infarctions are rare, probably because

the transit time of circulating blood across the coronary vessels is very short and negligible intravascular sickling takes place (101,102).

Bone and Joint Manifestations. Increased erythropoiesis leads to expansion of bone marrow in children with sickle cell disease. The most prominent manifestation of this is the widening of the diploe space in the skull vault, leading to protrusions (bossing) characteristically seen in the frontal and parietal bones. The maxilla is also quite often enlarged with protrusion of the upper teeth. In severe cases, there may be malocclusion of the jaw (103,104). These jaw abnormalities are referred to jointly as gnathopathy. Long bones show osteoporosis, widening of the medullary cavity, and thinning of the cortex. Avascular necrosis of the femoral head occurs in up to 30% of SS and SC patients by adulthood, but age of onset is usually in childhood and adolescence. The other common bone manifestation is acute infection (osteomyelitis) (105). In sickle cell disease the causative organism is *Salmonella* and multiple bone involvement is quite frequent (106).

Leg Ulceration. Chronic leg ulceration is common in sickle cell disease, mainly in adolescents and young adults. Associated factors include low social class, trauma, and poor nutrition. Typically it affects the area around the ankles, above the medial or lateral malleolus, because the blood supply in this region is of marginal adequacy even in normal individuals (107).

Physical and Sexual Development. Chronic anaemia and low endocrine production (especially growth hormone in childhood) are some of the factors causing delayed physical and sexual development in sickle cell disease (108-111). There is significant lower mean weight which often manifests within the first year of life and persists in all age groups. Mean height is also reduced in childhood; however, several studies have reported a catch-up period during the adolescent years when SS patients may overtake their AA counterparts in height. Puberty is delayed in both boys and girls, and menarche is also delayed in the latter (112-114). While there are no data supporting relative infertility among SS females, in male adolescents and adults there is a low level of testosterone, immaturity of seminiferous tubules, reduction in semen volume, sperm count, and motility (112).

Priapism. Sustained painful penile erection is a distressing complication of sickle cell disease, resulting from localised sickling and obstruction of venous drainage from the corpora cavernosa. Age of onset is usually in childhood. Major episodes are preceded by multiple, short-lasting ("stuttering") episodes (115). It may be spontaneous or follow sexual intercourse or masturbation. Major episodes, with or without treatment, are quite often followed by partial or complete impotence (116).

Pregnancy. Sickle cell disease is a high-risk factor in pregnancy. Acute hepatic and splenic sequestration crisis and pulmonary thrombo-embolism are two well-recognised complications in pregnancy. Megaloblastic anaemia from folate deficiency, bone pain crisis, toxæmia, and urinary tract infections are other common complications. Fetal wastage from abortions, stillbirths, and neonatal deaths are common, and low birth weight is characteristic (117,118).

Clinical Features in Adults. SS patients who survive into adulthood usually would have reduced frequency of painful crisis. However, unexplained cardiac failure and other end-organ damage (chronic renal and chronic hepatic disease) predominate in this period. Problems associated with impotence, especially in males who had suffered from priapism, and reproductive problems associated with pregnancy, in females, are encountered. Other common problems include gallbladder disease, cerebrovascular accidents (especially subarachnoid haemorrhage), and skeletal complications (avascular necrosis of the head of the femur).

MANAGEMENT

Since effective, definitive, and safe anti-sickling drug therapy is not yet available, the management of the disease is supportive and acute illness should be prevented as much as possible or managed as they occur. Overall management outlines are as follows:

Early Diagnosis and Good Follow-Up. There is convincing evidence that patients diagnosed and followed from the newborn period have better chances of survival than when diagnosis is delayed. All diagnosed patients should be followed in a sickle cell clinic that provides as much comprehensive care as possible and is manned by adequately trained personnel. The latter should be able to provide health education/genetic counselling and be able to recognise early warning signs of impending acute illness. At each clinic visit, baseline "steady state" physical and laboratory findings should be recorded. These data form the basis for comparison when the patient is seen with an acute illness. Regular ophthalmologic and audiologic studies, and gallbladder ultrasonographic scans should be scheduled as appropriate (119).

Health Education/Genetic Counselling. This entails the education of parents and patients in the nature of the disease, its inheritance pattern, symptomatology, complications, and prevention/treatment options. The need for continuous medical follow-up and good nutrition should be stressed. Counselling should not be directive, it should be simple and in a language that the client can understand. Decisions about marriage, childbearing, and other sensitive matters should be left to the patient and his/her parents/guardians, but all options should be discussed.

Prevention of Infections. Since bacterial infections are the leading cause of death, these should be prevented as much as possible. In many centres SS patients are started on oral penicillin prophylaxis in the newborn period and continued for the first 2 years of life. At 2 years, pneumococcal and *H. influenzae* vaccines are given (120-123). In the tropics where malaria is endemic, malaria chemoprophylaxis is routinely given.

Folate Supplementation. Folic acid (1-5 mg daily) is given to prevent megaloblastic crisis, although there is no real evidence for its necessity and efficacy. Some earlier reports suggested that folic acid improves physical and sexual development.

Management of Acute Illness. These should be diagnosed promptly and managed aggressively. This includes the management of acute crises, infections, and other complications.

Anti-Sickling Agents. A number of covalent and non-covalent compounds exhibit anti-sickling properties *in vitro* and several, *e.g.* urea and cyanate, have been tried clinically (124,125). However, they both cause significant toxicity and their use has not been encouraged.

Hyponatraemia. Low plasma osmolarity causes osmotic swelling of RBCs, with a decrease of intracellular Hb S concentration in SS patients. Hyponatraemia has been induced with a vasopressin analogue, desamino-D-arginine vasopressin (DDAVP) and restriction of sodium intake (126). Although this was associated with decrease in frequency of crisis in the few patients in whom it was tried, it is a difficult regimen to maintain.

Hb F Synthesis. Elevated Hb F levels are associated with decreased clinical severity in SS patients. Several cytotoxic drugs, *e.g.* hydroxyurea and 5-azacytidine, known to induce Hb F synthesis in adults are currently being tried in the management of SCA (127). Other pharmacologic agents, *e.g.* butyrate (128) and erythropoietin (129), have been used for the same purpose.

Bone Marrow Transplantation. This has proved to be the treatment of choice for certain haemoglobinopathies, especially β -thal major (Cooley's Anaemia) when a histocompatible sibling donor is available. Because SCA is more variable in its clinical severity, bone marrow transplantation has not been widely used. However, it is being increasingly encouraged for patients with a severe clinical course. The selection criteria include patients with neurological deficits, cerebrovascular accident or subarachnoid haemorrhage, two or more episodes of acute chest syndrome, recurrent and debilitating pain, while exclusion criteria include major intellectual impairment, portal fibrosis, renal impairment, severe chronic lung disease, and cardiomyopathy (131-133).

Gene Therapy. Considerable research is being directed at the possibility of gene therapy for the disease and this may become a viable option in the future (134,135). The current approach focuses on the transfer of a normal human β -globin gene into the patient's haemopoietic stem cell. The expression of the transferred sequence should be erythroid-specific and balance the expression of the endogenous α -globin genes as in normal adult erythropoiesis. The possibility of using recombinant adeno-associated virus (AAV) vectors for the transfer is being explored. Einerhand et al (136) have replaced the entire protein coding domain of AAV with a human β -globin gene, and the DNase HS-4, -3, and -2 of the β -globin gene LCR. Recombinant virus was produced by co-transfection of the construct with the helper plasmid pAAV/Ad into adeno-virus-infected 293 cells. The vector replicated to high titers and could efficiently transduce haemopoietic and non-haemopoietic cells. In transduced cells and G418 selected MEL cells clones of human globin expression was accurately regulated. Research along these lines show considerable promise for successful gene therapy in the near future.

REFERENCES

1. Herrick, J.B.: Peculiar elongated and sickle shaped red blood corpuscles in a case of severe anemia. *Arch. Intern. Med.*, 6:517, 1910.
2. Horton, J.A.B.: *The Diseases of Tropical Climates and Their Treatment*, Churchill, London, 1874.
3. Emmel, V.E.: A study of the erythrocytes in a case of severe anemia with elongated and sickle-shaped red blood corpuscles. *Arch. Intern. Med.*, 20:586, 1917.
4. Pauling, L., Itano, H.A., Sing, S.J., and Wells, I.C.: Sickle cell anemia, a molecular disease. *Science*, 110: 543, 1949.
5. Ingram, V.M.: A specific chemical difference between the globins of normal human and sickle cell anaemia haemoglobin. *Nature*, 178:792, 1956.
6. Perutz, M.F., Liguori, A.M., and Erlich, F.: X-ray and solubility studies of the haemoglobin of sickle cell anaemia patients. *Nature*, 167:929, 1951.
7. Eaton, W.A. and Hofrichter, J.: Hemoglobin S gelation and sickle cell disease. *Blood*, 70:1245, 1987.
8. Nagel, R.L.: Severity, epistatic effects and genetic markers in sickle cell anemia. *Semin. Hematol.*, 28:180, 1991.
9. Nagel, R.L.: Sickle cell anemia is a multigene disease: Sickle painful crises, a case in point. *Am. J. Hematol.*, 42:96, 1993.
10. Kaul, D.K. and Nagel, R.L.: Sickle cell vasoocclusion: Many issues and some answers. *Experientia*, 49:65, 1993.
11. Ueda, Y. and Bookchin, R.M.: Effects of carbon dioxide and pH variations *in vitro* on blood cell volume, transmembrane pH gradients and sickling in sickle cell anemia. *J. Lab. Clin. Med.*, 104:146, 1984.
12. Vitoux, D.O., Olivieri, R.P., and Garay, E.J.: Inhibition of K^+ efflux and dehydration of sickle cells by [(dihydroindenyl)oxy] alcanoic acid: An inhibitor of the K^+Cl^- cotransport system. *Proc. Natl. Acad. Sci. USA*, 86:4273, 1989.
13. Canessa, M., Fabry, M.E., Blumenfeld, N., and Nagel, R.L.: Volume-stimulated, Cl^- -dependent K^+ efflux is highly expressed in young human red cells containing normal hemoglobin or Hb S. *J. Membr. Biol.*, 97:97, 1987.
14. Bookchin, R.M., Balazs, T., and Landau, L.C.: Determinants of red cell sickling. Effects of varying pH and of increasing intracellular hemoglobin concentration by osmotic shrinkage. *J. Lab. Clin. Med.*, 87:597, 1976.
15. Hebbel, R.P., Boogaerts, M.A.B., Eaton, J.W., and Steinberg, M.H.: Erythrocyte adherence to endothelium in sickle cell anemia. A possible determinant of severity. *N. Engl. J. Med.*, 302:992, 1980.
16. Serjeant, G.R. and Milner, P.F.: The irreversibly sickled cell: A determinant of haemolysis in sickle cell anaemia. *Br. J. Haematol.*, 41:83, 1969.
17. Serjeant, G.R.: Nomenclature and genetics of sickle cell disease. In: *Sickle Cell Disease*, page 29, Oxford University Press, Oxford, 1985.
18. Huisman, T.H.J.: The structure and function of normal and abnormal haemoglobins. In: *The Haemoglobinopathies*, edited by D.J. Weatherall and D.R. Higgs, Bailliere's Clinical Haematology, Vol. 6, page 1, W.B. Saunders Company, London, 1993.
19. Antonarakis, S.E., Boehm, C.D., Serjeant, G.R., Theisen, C.E., Dover, G.J., and Kazazian, H.H., Jr.: Origin of the β^S gene in Blacks: The contribution of recur-

- rent mutation or gene conversion or both. *Proc. Natl. Acad. Sci. USA*, 81:853, 1984.
20. Nagel, R.L. and Ranney, H.M.: Genetic epidemiology of structural mutations of the β globin gene. *Semin. Hematol.*, 27:342, 1990.
 21. Nagel, R.L. and Fleming, A.F.: Genetic epidemiology of the β^S gene. In: *Epidemiology of Haematological Disease, Part II*, edited by A.F. Fleming, Bailliere's Clinical Haematology, Vol. 5, page 331, W.B. Saunders Company, London, 1992.
 22. Allison, A.C.: Protection afforded by sickle cell trait against subtertian malaria infection. *Br. Med. J.*, i:290, 1954.
 23. Fleming, A.F., Storey, J., Molineaux, L., Kagan, I., and Cornille-Brögger, R.: Abnormal haemoglobins in the Sudan savanna of Nigeria. I. Prevalence of haemoglobins and relationships between sickle cell trait, malaria and survival. *Ann. Trop. Med. Parasitol.*, 73:161, 1979.
 24. Fleming, A.F., Akintunde, A., Attai, E.D.E., Bradley-Moore, A.M., Greenwood, B.M., Bradley, A., Kirkwood, B.R., and Gilles, H.M.: Malaria and haemoglobin genotype in young northern Nigerian children. *Ann. Trop. Med. Parasitol.*, 78: 395, 1985.
 25. Tuan, D., Solomon, W., Li, Q., and London, I.M.: The " β -like-globin" gene domain in human erythroid cells. *Proc. Natl. Acad. Sci. USA*, 82:6384, 1985.
 26. Grosveld, F., Blom van Assendelf, G., Greaves, D.R., and Kollias, G.: Position-independent, high level expression of the human β -globin gene in transgenic mice. *Cell*, 51:975, 1987.
 27. Tuan, D., Solomon, W.B., London, I.M., and Lee, D.: An erythroid-specific developmental stage-dependent enhancer far upstream of the human " β -like-globin" genes. *Proc. Natl. Acad. Sci. USA*, 86:2554, 1989.
 28. Kan, Y.W. and Dozy, A.M.: Polymorphism of DNA sequence adjacent to the human β -globin structural gene: Relationship to the sickle mutation. *Proc. Natl. Acad. Sci. USA*, 75:5631, 1978.
 29. Kan, Y.W. and Dozy, A.M.: Evolution of the Hemoglobins S and C genes in world populations. *Science*, 209:388, 1980.
 30. Flint, J., Harding, R.M., Boyce, A.J., and Clegg, J.B.: The population genetics of haemoglobinopathies. In: *The Haemoglobinopathies*, edited by D.J. Weatherall and D.R. Higgs, Bailliere's Clinical Haematology, Vol. 6, page 215, W.B. Saunders Company, London, 1993.
 31. Lanclos, K.D., Oner, C., Dimovski, A.J., Gu, Y-C., and Huisman, T.H.J.: Sequence variations in the 5' flanking and IVS-II regions of the G_γ - and A_γ -globin genes of β^S chromosomes with five different haplotypes. *Blood*, 77: 2488, 1991.
 32. Oner, C., Dimovski, A.J., Olivieri, N.F., Schihiro, G., Codrington, J.F., Fattoum, S., Adekile, A.D., Oner, R., Yuregir, G.T., Altay, C., Gurgey, A., Gupta, R.B., Jogessar, V.B., Kitundu, M.N., Loukopoulos, D., Tamagnini, G.P., Ribeiro, M.L.S., Kutlar, F., Gu, L-H., Lanclos, K.D., and Huisman, T.H.J.: β^S Haplotypes in various world populations. *Hum. Genet.*, 89:99, 1992.
 33. Adekile, A.D., Kitundu, M.N., Gu, L-H., Adeodu, O.O., Lanclos, K.D., and Huisman, T.H.J.: Haplotypes in SS patients from Nigeria; characterization of one atypical β^S haplotype #19 (Benin) associated with elevated Hb F and high G_γ levels. *Ann. Hematol.*, 65:41, 1992.

34. Adekile, A.D.: Anthropology of the β^S gene-flow from West Africa to North Africa, the Mediterranean, and Southern Europe. *Hemoglobin*, 16:105, 1992.
35. Kulozik, A.E., Wainscoat, J.S., Serjeant, G.R., Kar, B.C., Al-Awamy, B., Esan, G.J.F., Falusi, A.G., Haque, S.K., Hilali, A.M., Kate, S., Ranasinghe, W.A.E.P., and Weatherall, D.J.: Geographical survey of the β^S globin gene haplotypes: Evidence for an independent Asian origin of the sickle cell mutation. *Am. J. Hum. Genet.*, 39:239, 1986.
36. Miller, B.A., Olivieri, N., Salameh, M., Ahmed, M., Antogenetti, G., Huisman, T.H.J., Nathan, D.G., and Orkin, S.H.: Molecular analysis of the high-Hemoglobin F phenotype in Saudi Arabian sickle cell anemia. *N. Engl. J. Med.*, 316:244, 1987.
37. Righetti, P.G., Gianazza, E., Bianchi-Bosisio, A., and Cossu, G.: Conventional isoelectric focusing and immobilized pH gradients for hemoglobin separation and identification. In: *The Hemoglobinopathies*, edited by T.H.J. Huisman, *Methods in Hematology*, Vol. 15, page 47, Churchill Livingstone, Edinburgh, 1986.
38. Shelton, J.B., Shelton, J.B., and Schroeder, W.A.: High performance liquid chromatographic separation of globin chains on a large-pore C_4 column. *J. Liq. Chromatogr.*, 7:1969, 1984.
39. Kutlar, F., Kutlar, A., and Huisman, T.H.J.: Separation of normal and abnormal hemoglobin chains by reversed-phase high-performance liquid chromatography. *J. Chromatogr.*, 357:147, 1986.
40. Huisman, T.H.J.: Introduction and review of standard methodology for the detection of hemoglobin abnormalities. In: *The Hemoglobinopathies*, edited by T.H.J. Huisman, *Methods in Hematology*, Vol. 15, page 1, Churchill Livingstone, Edinburgh, 1986.
41. Huisman, T.H.J.: Separation of hemoglobins and hemoglobin chains by high-performance liquid chromatography. In: *Separation of Biopolymers and Supramolecular Structures*, edited by Z. Deyl and M.T.W. Hearn, *J. Chromatogr.*, 418:277, 1987.
42. Huisman, T.H.J., Kutlar, F., and Gu, L-H.: γ Chain abnormalities and γ -globin gene rearrangements in newborn babies of various populations. *Hemoglobin*, 15:349, 1991.
43. Adekile, A.D., Gu, L-H., Baysal, E., Haider, M.Z., Al-Fuzae, L., Aboobacker, K.C., Al-Rashied, A., and Huisman, T.H.J.: Molecular characterization of α -thalassemia determinants, β -thalassemia alleles, and β^S haplotypes among Kuwaiti Arabs. *Acta Haematol.*, 92:176, 1994.
44. Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A.: Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, 239:487, 1988.
45. Gonzalez-Redondo, J.M., Stoming, T.A., Lanclos, K.D., Gu, Y.C., Kutlar, A., Kutlar, F., Nakatsuji, T., Deng, B., Han, I.S., McKie, V.C., and Huisman, T.H.J.: Clinical and genetic heterogeneity in Black patients with homozygous β -thalassemia from the Southeastern United States. *Blood*, 72:1007, 1988.
46. Yang, K.G., Kutlar, F., George, E., Wilson, J.B., Kutlar, A., Stoming, T.A., Gonzalez-Redondo, J.M., and Huisman, T.H.J.: Molecular characterization of β -globin gene mutations in Malay patients with Hb E- β -thalassaemia and thalassaemia major. *Br. J. Haematol.*, 72:73, 1989.

47. Yuregir, G.T., Aksoy, K., Curuk, M.A., Dikmen, N., Fei, Y.-J., Baysal, E., and Huisman, T.H.J.: Hb H disease in a Turkish family resulting from the interaction of a deletional α -thalassaemia-1 and a newly discovered poly A mutation. *Br. J. Haematol.*, 80:527, 1992.
48. Old, J.M., Thein, S.L., Weatherall, D.J., Cao, A., and Loukopoulos, D.: Prenatal diagnosis of the major hemoglobin disorders. *Mol. Biol. Med.*, 6:55, 1989.
49. Fischel-Gjodsian, N.: Prenatal diagnosis of hemoglobinopathies. *Clin. Perinatal.*, 17:811, 1990.
50. Alter, B.P.: Antenatal diagnosis. Summary of results. *Ann. N.Y. Acad. Sci.*, 612:237, 1990.
51. Serjeant, G.R.: Recognition of clinical features. In: *Sickle Cell Disease*, page 74, Oxford University Press, Oxford, 1985.
52. Higgs, D.R., Aldridge, B.E., Lamb, J., Clegg, J.B., Weatherall, D.J., Hayes, R.J., Grandison, Y., Lowrie, Y., Mason, K.P., Serjeant, B.E., and Serjeant, G.R.: The interaction of α -thalassemia and homozygous sickle cell disease. *N. Engl. J. Med.*, 306:1441, 1982.
53. Mears, J.G., Lachman, H.M., Labie, D., and Nagel, R.L.: α -Thalassemia is related to prolonged survival in sickle cell anemia. *Blood*, 62:286, 1983.
54. Steinberg, M.H., Rosenstock, W., Coleman, M.B., Adams, J.G., Platika, O., Cedeno, M., Rieder, R.F., Wilson, J.T., Milner, P., and West, S.: Effects of thalassemia and microcytosis on the hematologic and vaso-occlusive severity of sickle cell anemia. *Blood*, 63:1353, 1984.
55. Embury, S.H.: The interaction of α -thalassemia with sickle cell anemia. *Hemoglobin*, 12:509, 1988.
56. Adams, R.J., Kutlar, A., McKie, V., Carl, E., Nichols, F.T., Liu, J.C., McKie, K., and Clary, A.: Alpha thalassemia and stroke risk in sickle cell anemia. *Am. J. Hematol.*, 45:279, 1994.
57. Adekile, A.D., Liu, J.-C., Sulzer, A.J., and Huisman, T.H.J.: Frequency of the α -thalassemia-2 gene among Nigerian SS patients and its influence on malaria antibody titers. *Hemoglobin*, 17:73, 1993.
58. Goldberg, M.A., Husson, M.A., and Bunn, H.F.: The participation of Hemoglobins A and F in the polymerization of sickle hemoglobin. *J. Biol. Chem.*, 252:3414, 1977.
59. Smits, H.L., Oski, F.A., and Brody, J.I.: The hemolytic crisis of sickle cell anaemia: The role of G-6-PD deficiency. *J. Pediatr.*, 74:544, 1969.
60. Adekile, A.D. and Huisman, T.H.J.: The level of fetal hemoglobin in children with sickle cell anemia: The influence of gender, haplotype and α -thalassemia-2 trait. *Acta Haematol.*, 90:34, 1993.
61. Miyoshi, K., Kaneto, Y., Kawai, H., Ohchi, H., Niki, S., Hasegawa, K., Shirakami, A., and Yamano, T.: X-linked dominant control of F cells in normal adult life: Characterization of the Swiss type hereditary persistence of fetal hemoglobin regulated dominantly by gene(s) on X chromosomes. *Blood*, 72:1854, 1988.
62. Dover, G.J., Smith, K.D., Chang, Y.C., Purvis, S., Mays, A., Meyers, D.A., Sheils, C., and Serjeant, G.: Fetal hemoglobin levels in sickle cell disease and normal individuals are partially controlled by an X-linked gene located at Xp22.2. *Blood*, 80:816, 1991.

63. Morris, J., Dunn, D., Beckford, M., Grandison, Y., Mason, K., Higgs, D.R., De Ceulear, K., Serjeant, B.E., and Serjeant, G.R.: The haematology of homozygous sickle cell disease after the age of 40 years. *Br. J. Haematol.*, 77:382, 1991.
64. Bhaumik, K.: Fetal hemoglobin synthesis in sickle cell anemia: Some molecular considerations. *Am. J. Hematol.*, 46:101, 1994.
65. Steinberg, M.H., Hsu, H., Nagel, R.L., Milner, P.F., Adams, J.G., Benjamin, L., Fryd, S., Gillette, P., Gilman, J., Josifovska, O., Hellman-Erlingsson, S., Safaya, S., Huey, L., and Rieder, R.F.: Gender and haplotype effects upon hematological manifestations of adult sickle cell anemia. *Am. J. Hematol.*, 48:175, 1995.
66. Chang, Y.C., Smith, K.D., Moore, R.D., Serjeant, G.R., and Dover, G.J.: An analysis of fetal hemoglobin variations in sickle cell disease: The relative contributions of the X-linked factor, β -globin haplotypes, α -globin gene number, gender and age. *Blood*, 85:1111, 1995.
67. Powars, D.R.: Sickle cell anemia: β^S -gene cluster haplotypes as prognostic indicators of vital organ damage. *Semin. Hematol.*, 28:202, 1991.
68. Hattori, Y., Kutlar, F., Mosley, C.J., Mayson, S.M., and Huisman, T.H.J.: Association of the level of γ chain in the fetal hemoglobin of normal adults with specific haplotypes. *Hemoglobin*, 10:185, 1986.
69. Öner, C., Dimovski, A.J., Altay, Ç., Gurgey, A., Gu, Y.C., Huisman, T.H.J., and Lanclos, K.D.: Sequence variations in the 5' hypersensitive site-2 of the locus control region of β^S chromosomes are associated with different levels of fetal globin in Hemoglobin S homozygotes. *Blood*, 79:813, 1992.
70. Adekile, A.D., Dimovski, A.J., Öner, C., Lanclos, K.D., and Huisman, T.H.J.: Haplotype-specific sequence variations in the locus control region (5' hypersensitive sites 2, 3, 4) of β^S chromosomes. *Hemoglobin*, 17:475, 1993.
71. Berg, P.E., Mittelman, M., Elion, J., Labie, D., and Schechter, A.N.: Increased protein binding to a -530 mutation of the human β -globin gene associated with decreased β -globin synthesis. *Am. J. Hematol.*, 36:42, 1991.
72. Elion, J., Berg, P.E., Lapoumeroulie, C., Trabuchet, G., Mittelman, M., Krishna-moorthy, R., Schechter, A.N., and Labie, D.: DNA sequence variation in a negative control region 5' to the β -globin gene correlates with the phenotypic expression of the SS mutation. *Blood* 79:787, 1992.
73. Dimovski, A.J., Adekile, A.D., Divoky, V., Baysal, E., and Huisman, T.H.J.: Polymorphic pattern of the (AT) $_X$ (T) $_Y$ motif at -530 5' to the β -globin gene in over 40 patients homozygous for various β -thalassemia mutations. *Am. J. Hematol.*, 45:51, 1994.
74. Serjeant, G.R., Serjeant, B.E., Thomas, P.W., Anderson, M.J., Patou, G., and Pattison, J.R.: Human parvovirus infection in homozygous sickle cell disease. *The Lancet*, 341:1237, 1993.
75. Powars, D.R.: The natural history of sickle cell anemia: The first 10 years. *Semin. Hematol.*, 12:257, 1975.
76. Emond, A., Collis, R., Darvill, D., Higgs, D.R., Mande, G.H., and Serjeant, G.R.: Acute splenic sequestration in homozygous sickle cell disease: Natural history and management. *J. Pediatr.*, 107:201, 1985.
77. Leikin, S.L., Gallagher, D., Kinney, T.R., Sloane, D., Klug, P., and Wasima, R.: Mortality in children and adolescents with sickle cell disease. *Pediatrics*, 84: 500, 1989.

78. Barrett-Connor, E.: Bacterial infection and sickle cell anemia. An analysis of 250 infections in 168 patients and a review of the literature. *Medicine*, 50:97, 1971.
79. Pearson, H.A., Spencer, R.P., and Cornelius, E.A.: Functional asplenia in sickle cell anemia. *N. Engl. J. Med.*, 281:923, 1969.
80. Pearson, H.A., McIntosh, S., Ritchie, A.K., Lobel, J.S., Rooks, Y., and Johnston, D.: Developmental aspects of splenic function in sickle cell disease. *Blood*, 53:358, 1979.
81. Winkelstein, J.A. and Drachman, R.H.: Deficiency of pneumococcal serum opsonization activity in sickle cell disease. *N. Engl. J. Med.*, 279:459, 1968.
82. Dimitrov, N.V., Douwes, F.R., Bartalotta, B., Nochumson, S., and Toth, M.A.: Metabolic activity of polymorphonuclear leukocytes in sickle cell anemia. *Acta Haematol.*, 47:283, 1972.
83. Ebong, W.W.: Acute osteomyelitis three years after a closed fracture in an adult with sickle cell anemia. A case report. *J. Bone Joint Surg.*, 62A:1196, 1980.
84. Adekile, A.D., McKie, M.M., Adeodu, O.O., Sulzer, A.J., McKie, V.C., Kutlar, F., Ramachandran, M., Kaine, W., Akenzua, G.I., Akolo, A.A., Asindi, A.A., Obinyan, E.A., Ogala, W.N., Ibrabim, M., and Huisman, T.H.J.: The spleen in sickle cell anemia: Comparative studies of Nigerian and U.S. patients. *Am. J. Hematol.*, 42:316, 1993.
85. Rosenblate, H.J., Eisenstein, R., and Holmes, A.W.: The liver in sickle cell anemia. *Arch. Pathol.*, 90:235, 1980.
86. Adekile, A.D.: Experience with cholelithiasis in Nigerian sickle cell patients. *Am. J. Pediatr. Hematol./Oncol.*, 7:261, 1985.
87. Cetta, F.: The role of bacteria in pigment gallstone disease. *Ann. Surg.*, 213:315, 1991.
88. Statius van Eps, L.W., Pinedo-Veels, C., De Vries, G.H., and De Konig, J.: Nature of concentrating defect in sickle cell nephropathy. Microangiographic studies. *The Lancet*, i:450, 1970.
89. DeJong, P.E. and Statius van Eps, L.W.: Sickle cell nephropathy: New insights into its pathophysiology. *Kidney Int.*, 27:711, 1985.
90. Cruz, I.A., Hosten, A.O., and Dillard, M.G.: Advanced renal failure in patients with sickle cell anemia: Clinical course and prognosis. *J. Natl. Med. Assoc.*, 74:1103, 1982.
91. Izuora, G.I., Kaine, W.N., and Emodi, I.: Neurological disorders in Nigerian children with homozygous sickle cell anaemia. *E. Afr. Med. J.*, 66:653, 1989.
92. Ohene-Frempong, K.: Stroke in sickle cell disease: Demographic, clinical and therapeutic considerations. *Semin. Hematol.*, 28:213, 1991.
93. To, K.W. and Nadel, A.J.: Ophthalmologic complications in hemoglobinopathies. *Hematol. Oncol. Clin. N. Am.*, 5:535, 1991.
94. Odetoyinbo, O. and Adekile, A.D.: Sensorineural hearing loss in children with sickle cell anemia. *Ann. Oto. Laryngol.*, 96:258, 1987.
95. Goldberg, M.F.: Classification and pathogenesis of proliferative sickle retinopathy. *Am. J. Ophthalmol.*, 71:649, 1971.
96. Davies, S.C., Luce, P.J.H., Winn, A.A., Riordan, J.F., and Brozovic, M.: Acute chest syndrome in sickle cell disease. *The Lancet*, i:36, 1984.

97. Barrett-Connor, E.: Acute chest pulmonary disease and sickle cell anemia. *Am. Rev. Respir. Dis.*, 104:159, 1987.
98. Vichinsky, E., Williams, R., Das, M., Earles, A.N., Lewis, N., Adler, A., and McQuitty, J.: Pulmonary fat embolism: A distinct cause of severe acute chest syndrome in sickle cell anemia. *Blood*, 83:3107, 1994.
99. Haynes, J., Jr. and Kirkpatrick, M.B.: Pneumonia and pulmonary infarction in sickle cell anemia. *JAMA*, 224:997, 1973.
100. Koren, A., Wald, I., Halevi, R., and Ben Ami, M.: Acute chest syndrome in children with sickle cell anemia. *Pediatr. Hematol. Oncol.*, 7:99, 1990.
101. Lindsay, J., Jr., Meshel, J.C., and Patterson, R.H.: The cardiovascular manifestations of sickle cell disease. *Arch. Intern. Med.*, 133:643, 1974.
102. Falk, R.H. and Hood, W.B.: The heart in sickle cell anemia. *Arch. Intern. Med.*, 142:1680, 1982.
103. Sebes, J.I. and Diggs, L.W.: Radiographic changes of the skull in sickle cell anemia. *Am. J. Roentgenol.*, 132:373, 1979.
104. Sebes, J.I. and Kraus, A.P.: Avascular necrosis of the hip in the sickle cell hemoglobinopathies. *J. Canad. Assoc. Radiol.*, 34:136, 1983.
105. Adeyokunnu, A.A. and Hendrickse, R.G.: Salmonella osteomyelitis in childhood. A report of 63 cases seen in Nigerian children of whom 57 had sickle cell anaemia. *Arch. Dis. Child.*, 55:175, 1980.
106. Mankad, V.N., Williams, J.P., Harpen, M.D., Mancini, E., Longnecker, G., Moore, R.B., Shah, A., Yang, Y.M., and Brogden, B.G.: Magnetic resonance imaging of bone marrow in sickle cell disease: Clinical, hematologic and pathologic correlations. *Blood*, 75:274, 1989.
107. Koshy, M., Entshuah, R., Koranda, A., Kraus, J.P., Johnson, R., Bellvue, R., Flournoy-Gill, Z., and Levy, P.: Leg ulcers in patients with sickle cell disease. *Blood*, 74:1403, 1989.
108. Olanbiwonnu, N.P., Penny, R., and Frasier, S.D.: Sexual maturation in subjects with sickle cell anemia: Studies of gonadotrophin concentration, height, weight and skeletal age. *J. Pediatr.*, 87:459, 1975.
109. Odonkor, P.O., Addae, S.K., and Apatu, R.S.: Basal metabolic rate and serum thyroid hormone levels in adolescent sickle cell patients. *IRCS Med. Sci.*, 10: 8981, 1982.
110. Modebe, O. and Ifenu, S.A.: Growth retardation in homozygous sickle cell disease: Role of calorie intake and possible gender-related differences. *Am. J. Hematol.*, 44:149, 1993.
111. Wethers, D.L.: Delayed growth and sexual maturation in sickle cell disease. *Ann. N.Y. Acad. Sci.*, 565:137, 1989.
112. Orbeleas, D.: Gonadal function abnormalities in sickle cell anemia. Studies in adult male patients. *Ann. Intern. Med.*, 85:601, 1976.
113. Dada, O.A. and Nduka, E.U.: Endocrine function and haemoglobinopathies: Relationship between sickle cell gene and circulating plasma levels of testosterone, luteinising hormone and follicle stimulating hormone in adult males. *Clin. Chim. Acta*, 105:269, 1980.
114. Adekile, A.D., Okonofua, F.E., and Durosini, M.E.: Pattern of menstruation in sickle cell anaemia patients. *Nig. Med. J.*, 20:26, 1990.
115. Adogu, A.A.: Stuttering priapism in sickle cell disease. *Br. J. Urol.*, 67:105, 1991.

116. Fowler, J.E., Koshy, M., Strub, M., and Chinn, S.K.: Priapism associated with sickle cell hemoglobinopathies: Prevalence, natural history and sequelae. *J. Urol.*, 145:65, 1991.
117. Hendrickse, J.P. deV., Harrison, K.A., Watson-Williams, E.J., Luzzatto, L., and Ajabor, L.N.: Pregnancy in homozygous sickle cell anaemia. *J. Obstet. Gynaecol. Br. Commun.*, 79:396, 1972.
118. Koshy, M. and Burd, L.: Management of pregnancy in sickle cell syndromes. *Hematol. Oncol. Clin. N. Am.*, 5:585, 1991.
119. Vichinsky, E.P.: Comprehensive care in sickle cell disease: Its impact on morbidity and mortality. *Semin. Hematol.*, 28:220, 1991.
120. Sarnaik, S., Kaplan, J., Schiffman, G., Bryla, D., Robbins, J.B., and Schneerson, R.: Studies on *Pneumococcus* vaccine alone or mixed with DTP and on *Pneumococcus* type 6B and *Haemophilus influenzae* type B capsular polysaccharide-tetanus toxoid conjugate in two-to-five-year old children with sickle cell anemia. *Pediatr. Infect. Dis.*, 9:181, 1990.
121. Wong, W-Y., Overturf, G.D., and Powars, D.R.: Infection caused by *Streptococcus pneumoniae* in children with sickle cell disease: Epidemiology, immunologic mechanisms, prophylaxis and vaccination. *Clin. Infect. Dis.*, 14:1124, 1992.
122. Gigliotti, F., Feldman, S., Wang, W.C., Day, S.W., and Brunson, G.: Immunization of young infants with sickle cell disease with a *Haemophilus influenzae* type B polysaccharide-diphtheria CRM197 protein conjugate vaccine. *J. Pediatr.*, 114:1006, 1989.
123. Gaston, M.H., Verter, J.I., Woods, G., Pegelow, C., Kelleher, J., Presbury, G., Zarkowsky, H., Vichinsky, E., Iyer, R., Lobel, J.S., Diamond, S., Holbrook, C.T., Gill, F.M., Ritchey, K., and Falletta, J.M.: Prophylaxis with oral penicillin in children with sickle cell anemia. A randomized trial. *N. Engl. J. Med.*, 314:1593, 1986.
124. Gail, M., Beach, J., Dark, A., Lewis, R., and Morrow, H.: A double-blind randomized trial of low-dose oral urea to prevent sickle cell crisis. *J. Chronic Dis.*, 35:151, 1982.
125. Gillette, P.N., Manning, J.M., and Cerami, A.: Increased survival of sickle cell erythrocytes after treatment *in vitro* with sodium cyanate. *Proc. Natl. Acad. Sci. USA*, 68:2791, 1971.
126. Rosa, R.M., Bierer, B.E., Thomas, R., Stoff, J.S., Kruskall, M., Robinson, S., Bunn, H.F., and Epstein, F.H.: A study of induced hyponatremia in the prevention of sickle cell crisis. *N. Engl. J. Med.*, 303:1138, 1980.
127. Stamatoyannopoulos, G. and Nienhuis, A.W.: Therapeutic approaches to hemoglobin switching in the treatment of hemoglobinopathies. *Ann. Rev. Med.*, 43:497, 1992.
128. Perrine, S.P., Miller, B.A., Faller, D.V., Cohen, R.A., Vichinsky, E.P., Hurst, D., Lubin, B.H., and Papayannopoulos, Th.: Sodium butyrate enhances fetal globin gene expression in erythroid progenitors of patients with Hb SS and β thalassemia. *Blood*, 74:454, 1989.
129. Stamatoyannopoulos, G., Umemura, T., Al-Khatti, A., Spadaccino, E., Abels, R.I., Fritsch, E.F., Papayannopoulos, Th.: Modulation of Hb F production by erythropoietin. *Prog. Clin. Biol. Res.*, 316:269, 1989.

130. Dover, G.J., Brusilov, S., and Charache, S.: Induction of fetal hemoglobin production in subjects with sickle cell anemia by oral sodium phenylbutyrate. *Blood*, 84:339, 1994.
131. Vermynen, C., Cornu, G., Philippe, M., Ninane, J., Borja, A., Latinne, D., Ferant, A., Michaux, J.L., and Sokal, G.: Bone marrow transplantation in sickle cell anaemia. *Arch. Dis. Child.*, 66:1195, 1991.
132. Kirkpatrick, D.V., Barrios, N.J., and Humbert, J.H.: Bone marrow transplantation for sickle cell anemia. *Semin. Hematol.*, 28:240, 1991.
133. Apperley, J.F.: Bone marrow transplant for the hemoglobinopathies. In: *Epidemiology of Haematological Disease, Part II*, edited by A.F. Fleming, Bailliere's Clinical Haematology, Vol. 5, page 229, W.B. Saunders Company, London, 1992.
134. Steinberg, M.H.: Prospects for gene therapy for hemoglobinopathies. *Am. J. Med. Sci.*, 302:298, 1991.
135. Levine, F.: Gene therapy. *Am. J. Dis. Child.*, 147:1167, 1993.
136. Einerhand, M.P.W., Antoniou, M., Zoloturkhin, S., Muzyezka, N., Berns, F., Grosveld, F., and Valerio, D.: Regulated high level human β -globin gene expression in erythroid cells following rAAV-mediated gene transfer. Abstract, 20th Annual Meeting of the National Sickle Cell Disease Program, Boston, page 19, 1995.

CHAPTER 2

MATERIALS AND METHODS

CHAPTER 2

1. **Materials and Methods.** This chapter provides general information on the patients and experimental techniques (1) used in these studies. Detailed descriptions of the methodology are provided in Chapters 3 and 4.

2. **Patients.** As many as 330 patients from 297 unrelated Nigerian families were recruited for various aspects of the studies. Some of their AS and AA relatives were also studied as controls. These patients were being followed in the paediatrics departments of various teaching hospitals in the country. These hospitals are based in the following cities: Ile-Ife, Benin, Enugu, Calabaar, Sokoto, Kaduna, and Maiduguri. About 30 Kuwaiti patients have been studied; these patients are being followed mainly in the two teaching hospitals in the country: Mubarak Al-Kabeer and Al-Amiri. The patients were consecutive patients seen in the clinic at the time of the study. Informed consent was obtained from all the parents of the patients and from the patients where appropriate. Their charts and hospital records were reviewed to document the common causes of acute illness. Physical examination was carried out to document the associated clinical features and complications of SS disease. Blood was taken from each patient for haematological and DNA studies. Although the diagnosis of SS disease was made in each of the hospitals by Hb electrophoresis, this was reconfirmed in the laboratory in Augusta, GA, USA.

3. **Blood Collection and Shipment.** About 5-10 ml of blood was obtained by venepuncture into vacutainers (Becton Dickinson Vacutainer Systems, New Jersey, N.J., USA) with EDTA as anticoagulant. The samples that were later analyzed in Augusta were stored at 4°C until the time of shipment. Samples were sent on ice by fast courier (or carried by the author) and usually arrived in Augusta within 4 days. Some samples were received more than 3 weeks after collection.

4. **Hb and Haematological Analyses**

4/a. **Haematological Data.** CBCs and red cell indices were obtained with a fully automated cell counter (Sysmex CC-620; Toa Medical Electronics Co., Kobe, Japan, or Coulter MAXM Analyzer; Coulter Electronics, Ltd., Luton, Bedfordshire, England).

4/b. **Hb Analyses.** RBCs were washed three times with isotonic saline and haemolysed with 1.5 volumes of water and 0.4 times the volume of CCl_4 for 15 minutes at room temperature with occasional stirring. Stroma was removed by centrifugation at 20,000 g at 4°C for 20 minutes. These haemolysates were used for electrophoretic and chromatographic analyses.

i. **IEF.** The IEF (2) analysis was performed for initial identification using commercially available agarose gels and buffers (Isolab, Inc., Akron, OH, USA). Usually, 6 μl of haemolysate was applied onto a gel and electrophoresis was per-

formed for 2 hours at 30 W constant power using an LKB Multiphor II Horizontal Unit (Pharmacia LKB, Uppsala, Sweden). When all Hbs focused at their respective pls, the gel was fixed for about 2 hours in 10% trichloroacetic acid for 10 minutes and the bands were visualised with a haem specific dye (o-dianisidine) and H_2O_2 . Band identification was by comparing the distances of migration to control samples containing the known Hb types of A, F, S, C, and Bart's (Fig. 2/1).

ii. **Foetal Hb Quantitation.** This was done by alkali denaturation and/or by two recently developed HPLC procedures.

iii. **Alkali Denaturation Method.** This is based on the higher resistance of Hb F to denaturation in alkali (3). The method involves the conversion of cyan-metHb before denaturation which prevents false-positives, particularly in patients with increased concentrations of the alkali-resistant CO-Hb, e.g. in heavy smokers. The method offers a reasonable accuracy for Hb F levels between 5-20%. The disadvantage is that Hb F levels below 5% are determined too high and those with levels above 20% are determined too low (4).

4/c. **HPLC Procedures.** There are two HPLC methods that offer high accuracy in the determination of Hb F levels. The first uses a weak cation exchange resin that allows complete separation of Hb F (as Hb F₀ and Hb F₁) from other types in less than 50 minutes (4,5). The second is a reversed phase method that allows the separation of haem and various globin chains (6,7).

i. **Cation Exchange HPLC.** Quantitation of Hb F was performed on a polyCAT A cation exchange column (4.6 x 200 mm with a particle size of 5 μm ; PolyLC, Columbia, MD, USA) using a Waters HPLC system (Waters Chromatography Division, Milford, MA, USA). The developers used are as follows: Developer A: 35 mM Bis-Tris, 3 mM NH_4 -acetate, 1.5 mM KCN, pH 6.47; developer B: 35 mM Bis-Tris, 1.5 mM KCN, 150 mM Na-acetate, 16.85 mM NH_4 -acetate, pH 7.0. The column was initially purged for 5 minutes with 100% developer B and equilibrated with 25% developer B for 10 minutes. Some 50-200 μg Hb (10-15 μl haemolysate) was applied to the column and the chromatogram developed with a gradient of 25% developer A to 85% developer B in 85 minutes with a flow-rate of 0.8 ml/min. The absorbance of the effluent was continuously recorded at 415 nm with a chart speed of 0.25 cm/min. The order of elution of different types of Hbs is F, A, A₂, S, and C, that are readily separated from each other and accurately quantitated. A disadvantage is the minor contamination of Hb A₂ with Hb S₁ that results in slightly higher Hb A₂ values in the presence of Hb S.

ii. **Reversed Phase HPLC.** A large-pore Vydac C₄ column (Vydac, Hesperia, CA, USA) and two water-acetonitrile-TFA developers were used for the separation of globin chains as originally described by Shelton et al (6) and Kutlar et al (7). Developer A is 60% acetonitrile, 0.1% TFA, 80% water. The column was initially purged with 100% developer A and equilibrated for 10 minutes with 50% developer A and 50% developer B. Some 50-800 μg Hb in red cell lysates was applied to the column and the chromatogram developed in a gradient of 50% developer A to 70% developer B in 70 minutes at a flow-rate of 1 ml/min. The absorbance

of the effluent was continuously recorded at 215 nm and peaks are quantitated (as area %) by a data module. The separation of the globin chains by this method depends primarily on differences in their hydrophobicities. As a general rule, the more polar (less hydrophobic) chains elute earlier in the chromatogram (6). The presence of TFA in the developer results in a low pH of 2 that causes the dissociation of the Hbs in haem and the chains. The order of elution of the different Hb chains is β^C , β^S , β^A , δ , α , A_{γ}^T , G_{γ} , and A_{γ}^I . The levels of Hb F ($= G_{\gamma} + A_{\gamma}^I + A_{\gamma}^T$) are calculated as % of total β -like chains ($\delta + \beta + A_{\gamma}^T + G_{\gamma} + A_{\gamma}$). Slightly higher values of Hb F obtained by this method are perhaps due to some contamination of G_{γ} with α chains (7).

iii. **Hb F Composition Analysis.** The chain composition of Hb F was studied by the reversed phase HPLC method described above and also by the visual inspection of polyacrylamide gels (8). Samples that contain less than 20% Hb F in their red cell lysates (as estimated by IEF) required an initial enrichment of the Hb F fraction, which is performed by anion exchange DEAE-cellulose chromatography (9). The resin was equilibrated with repeated washing with a 0.2 M glycine, 15 mM KCN buffer, pH 7.5, and the chromatogram was developed using a 0.2 M glycine, 0.01% KCN, 0.02 M NaCl developer. Depending upon the % Hb F present in the red cell lysate, 20-60 mg Hb in the haemolysate were applied to a column (30 x 1.5 cm); the chromatogram was developed for 15 minutes at a flow-rate of 50 ml/hour. The Hb F fraction located at the top was cut from the column, poured into a microcolumn (7 x 0.5 cm) and eluted with a stripping buffer (0.01% KCN, 0.2 M NaCl, 0.2 M glycine).

iv. **Quantitation of Hb A₂.** In several instances, the Hb A₂ level was determined by anion exchange DEAE-cellulose chromatography using the Quick-Sep Hb A₂ Test System (Isolab, Inc., Akron, OH, USA). Some 5-10 μ l haemolysate were applied onto a column and the Hb A₂ was eluted from the column under specific conditions of pH and chloride ion concentration (developer A: 0.2 M glycine, 0.01% KCN). All the remaining Hbs were eluted from the column as a single fraction with developer B (0.2 M glycine, 0.01% KCN, 0.2 M NaCl). Absorbance values of the two fractions were measured at 415 nm. The disadvantage of the method is that Hbs E and C co-elute with Hb A₂.

5. DNA Analyses

5/a. **Isolation of Human Genomic DNA From Whole Blood.** DNA was isolated from white cells using the method of Poncz et al (10). About 5-10 ml whole blood was washed twice with 40 ml 1X reticulocyte saline (140 mM NaCl, 4 mM KCL, 6.8 mM MgCl₂) and the cells were pelleted by centrifugation at 2,500 g for 10 minutes at 4°C. Next, the RBCs were haemolysed with 20 ml of freshly prepared lysing buffer (131 mM NH₄Cl, 0.9 mM (NH₄)₂CO₃, pH 6.5) at room temperature for 20 minutes, with occasional shaking. After centrifugation at 500 g and 4°C, the supernatant (haemolysate) was collected and stored at -20°C for Hb analysis. The lysing procedure was repeated once more, and the pellet, consisting mainly of WBCs, was re-suspended in 10 ml STE buffer (0.1 NaCl, 0.05 M Tris-HCl, pH 7.4, 1 mM EDTA), 1 ml 10% SDS, and 0.1 ml proteinase K (10 mg/ml in 10 mM Tris-HCl, pH 7.5, 1 mM EDTA) and incubated overnight at 37°C without shaking. Deproteinisation was accomplished by phenol/chloroform/isoamyl alcohol extractions as follows: One

volume of phenol, saturated with 20 mM Tris, pH 8.0, containing 0.1 % hydroxyquinoline was added to the viscous solution, and the mixture was gently shaken for 30 minutes at room temperature. After centrifugation at 2,500 g and at 4°C, the upper aqueous phase was transferred to a second tube and the procedures were repeated. An equal volume of chloroform/isoamyl alcohol (19:1 by vol/vol) was added and the mixture was shaken gently for 10 minutes at room temperature. After centrifugation for 10 minutes at 4°C, the upper aqueous phase is transferred to a fresh tube and the DNA was precipitated with 5 volumes of ice-cold absolute ethanol. The DNA precipitate was transferred to a 1.5 ml Eppendorf tube with a sterile Pasteur pipette, washed twice with 70% ethanol, dried under vacuum, and dissolved by incubation in 1 ml TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) overnight at 37°C. The concentration of the DNA was determined from its extinction coefficient at 260 nm (1 OD = 50 µg/ml DNA). The DNA is stored at 4°C.

5/b. Oligonucleotide Synthesis. Synthetic oligonucleotides were used as amplification and sequencing primers in amplification (PCR) and DNA sequencing analysis, and as specific probes in the dot-blot hybridisation analysis. The oligonucleotides were synthesised by a solid-phase oligonucleotide synthesis method (11) on an Applied BioSystems 380B DNA synthesiser (Applied BioSystems, Inc., Foster City, CA, USA) in the laboratory in Augusta, GA, USA). Following synthesis, the oligonucleotides were removed from the solid support by an ammonia treatment (55°C) overnight, dried under vacuum, and purified by G-50 Sephadex molecular sieve chromatography using TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) elution buffer.

5/c. PCR. PCR is based on *in vitro* amplification of a specific DNA fragment flanked by two synthetic oligonucleotide primers complementary to the opposite strand of the fragment of interest (12). Repeated cycles of heat denaturation, annealing of the primers to the complementary sequences, and extension of the annealed primers with DNA polymerase lead to an exponential accumulation of the target fragment (10^6 - 10^7 copies). The use of programmable thermo-cyclers and heat-stable DNA polymerase makes it possible to automate the procedure (13). In this study, the PCR method was used for: 1) Generation of large amounts of specific double-stranded DNA fragments for dot-blot analysis, 2) generation of large amounts of specific single-stranded DNA fragments for DNA sequencing analysis, and 3) detection of deletional types of α -thal ($-\alpha^{3.7}$ and $-\alpha^{4.2}$ kb deletions; $-\text{MED-1}/\alpha\alpha$ deletion).

In general, the reaction was performed in three phases using the Perkin-Elmer-Cetus Automated Thermalcycler (Perkin Elmer Cetus, Norwalk, CT, USA). In the first phase, a reaction mixture was prepared containing 1 µg of genomic DNA, 20 mM MOPS, pH 7.8, 50 mM NaCl, 2.5 MgCl₂, 200 µM of each dNTP, and 100 pM of each of the oligonucleotide primers in a total volume of 100 µl. The genomic DNA was initially denatured at 99°C for 6 minutes. The temperature was decreased to 85°C and 2.5 U Taq polymerase was added to each sample. Samples were overlaid with 2-3 drops of mineral oil to prevent evaporation. In the second phase, the samples are subjected to 30-35 repeated cycles of denaturation at 95°C for 1 minute, annealing of the primers at 58-62°C for 1.5 minutes, and extension of the annealed primers at 72°C for 2.5-5 minutes. The temperature of annealing and the time of the primer extension depended on the sequences of the primers used and the length of the region

to be amplified, respectively. The third phase was the prolonged incubation (up to 10 minutes) at 72°C for the complete extension of all DNA strands synthesised in the previous steps. Following the reaction, a small aliquot (5 µl) of each sample was run on a 1.5% agarose gel in the presence of ethidium bromide (10 µg/ml) along with a *Hind III*-digested λ-DNA as a size marker; the efficiency of the amplification was inspected under UV light. The samples were kept at 4°C for further analyses.

5/b. Dot-Blot Hybridisation Analyses. The dot-blot hybridisation procedure was used for the detection of single nt substitutions (mutations and polymorphisms) in various regions of the β-globin gene cluster studied. An aliquot (40 µl) of PCR amplified DNA was denatured with 0.04 M NaOH and blotted in duplicate onto a nylon membrane (Zeta Probe Nylon Membrane; Bio-Rad) under vacuum using a spotting device (Bio-Dot; Bio-Rad). The DNA was fixed to the membranes by baking in a vacuum oven at 80°C for 2 hours. Two allele-specific oligonucleotide probes, both 19 nts long, were synthesised for each of the mutations studied; one (normal) was complementary to the sequence, and the other (mutant) with an identical sequence except for the substitution that was usually positioned in the centre of the probe. The normal and the mutant probes were end-labelled at the 5' end to a high activity with γ-³²P-dATP (~7,000 Ci/mM, 160 mCi/ml; ICN Biochemicals, Inc., Irvine, CA, USA) by T₄ polynucleotide kinase [United States Biochemical (USB), Cleveland, OH, USA] for 1-2 hours at 37°C. The unincorporated nt was removed from the probes using either G-50 Sephadex molecular sieve chromatography with TE elution buffer or by commercially available Nensorb (NEN) columns (NEN Products, DuPont, Boston, MA, USA) under the conditions recommended by the manufacturer. The activity of the probes as cpm was determined in a scintillation counter (Beckman, Palo Alto, CA, USA). For some of the experiments, instead of radioactive γ-³²P, an ECL 3'-oligo-labelling technique was used. This involves horseradish peroxidase catalysed oxidation of luminol to detect the presence of oligonucleotides tailed at the 3' end with fluorescein-11-dUTP (FI-dUTP, hybridised to target sequences on membranes (Amersham International, Co. Plc., Amersham, Buckinghamshire, UK).

The duplicate membranes were pre-hybridised for 30 minutes at 55°C in separate, sealed plastic bags, with 10 ml pre-hybridisation solution containing 5 x SSPE, 5 x Denhardt's, 0.5% SDS (20 x SSPE = 3.6 M NaCl, 20 mM NaH₂PO₄, 20 mM Na₂EDTA, pH 7.4; 50 x Denhardt's = 1% Ficoll, 1% PVP, 1% BSA). Following pre-hybridisation, the probe (50-100 x 10⁶ cpm) was added to a particular membrane, and the hybridisation was performed in a circulating water-bath for 1-2 hours. The temperature of hybridisation varied for each probe, depending on the T_m of the probe which can be calculated from the formula: $T_m = 4 \times (G + C) + 2 \times (A + T)$. Following hybridisation, the membrane was washed twice for 10 minutes at room temperature with 2 x SSPE, 0.1% SDS solution. The membranes were washed with TMAC washing solution (3.0 M TMAC, 50 mM Tris-HCl, pH 8.0, 2.0 mM EDTA, 0.1% SDS) for 20 minutes at 42°C, followed by a second wash at 58°C for 30 minutes. The use of TMAC in a washing solution equilibrates the A-T and G-C bonding strength differences, thus allowing the use of the same temperature for stringent washes for probes of the same size. After washing, the membranes were blotted dry, wrapped in plastic film, and autoradiographed by exposure to Kodak X-ray film (Eastman Kodak, Co., Rochester, N.Y., USA) for 2-12 hours at -70°C.

5/e. **DNA Sequencing.** DNA sequencing was used in the present study for the characterisation of the β -thal mutation in a few families reported in Chapter 4. The analysis was performed on single-stranded, PCR-amplified, DNA templates with the dideoxy chain termination method (14) using a Sequenase Version 2.0 DNA Sequencing Kit (USB). DNA sequencing comprises three phases: i) Synthesis and purification of single-stranded templates, ii) sequencing reaction, and iii) denaturing PAGE.

i. **Synthesis and Purification of Single-Stranded DNA Templates.** Amplification of single-stranded DNA templates is performed using similar methodology as described in Section 5/d for double-stranded PCR amplification except for an unbalanced ratio of the amplification primers (100 pM to 1 pM). The amplified DNA is purified by precipitation with 1 volume of 2.5 M ammonium acetate and 2 volumes of ice-cold ethanol at room temperature for 15 minutes. The mixture is centrifuged in a micro-centrifuge at 4°C for 15 minutes, and the resulting pellet washed 4-5 times with 70% ethanol. The pellet is dried under vacuum and dissolved in 10 μ l of water. One μ l of DNA solution is electrophoresed on a 1.5% agarose gel along with 0.3 μ g M-13 control single-strand DNA for monitoring the recovery and for an estimation of the DNA concentration.

ii. **Sequencing Reactions.** In general, sequencing reactions are performed following the protocol recommended for the use of the Sequenase Version 2.0 DNA Sequencing Kit (USB). All reactions are done in sterile 1.5 ml Eppendorf capped tubes. The reaction involves three steps: a) Annealing, b) labelling, and c) termination.

a. **Annealing reaction:** 1 μ L (50 pM) of sequencing primer and 2 μ L of 5X reaction buffer were added to the denatured DNA sample, up to a total of 10 μ L. The reaction mixture was vortexed briefly, centrifuged, and incubated at 37°C for 15 minutes.

b. **Labelling reaction:** The labelling mix provided in the sequencing kit was diluted 10 times with sterile water. The sequenase enzyme (13 U/ μ L) was then diluted (1:8) in Enzyme Dilution Buffer (USB) up to a total volume of 2 μ L. Next, 0.5 μ L of $\alpha^{35}\text{S}$ -dATP (800 Ci/mM, 10.0 mCi/ml; DuPont) were added to the diluted enzyme. All the dilutions were performed on ice. The labelling reaction was started with the addition of 1 μ L 0.1 M DTT, 1 μ L diluted Labelling Mix, and 2.3 μ L of diluted enzyme/ $\alpha^{35}\text{S}$ -dATP mixture to the tube containing the annealed template/primer. The sample was incubated at room temperature for 2 minutes.

c. **Termination reaction:** 2.5 μ L of each Termination Mixture (ddATP, ddCTP, ddGTP, and ddTTP) were placed in clean 1.5 ml Eppendorf tubes labelled A, C, G, and T, respectively. The tubes were pre-warmed for at least 1 minute at 37°C. When the labelling reaction was completed, 3.5 μ L of the reaction mix was added to each of the labelled tubes and incubated at 37°C for 5 minutes. The reactions were stopped with 4 μ L of Stop Solution and the tubes were kept on ice until loaded onto the gel.

iii. **Denaturing PAGE.** The sequencing reactions were separated on a 0.04 mm thick 7 M urea/8% polyacrylamide/TBE gel using a vertical electrophoresis apparatus for DNA sequencing (Model S2; BRL-GIBCO) and a high voltage constant power supply (2297-Microdrives; LKB-Broma, Uppsala, Sweden). The gel was allowed to polymerise for at least 1 hour, and next was pre-electrophoresed at 70 W constant power (1400-1500 V, 50-60 mA) for at least 30 minutes. The DNA samples were denatured for 3 minutes at 80°C, placed on ice for 3-5 minutes, and loaded onto the gel (2-3 μ L per well). The gel was run at constant power (75 W) for 1.5 to 5 hours, depending upon the proximity of the desired sequence to the sequencing primer. Following electrophoresis, the gel was fixed in a 10% acetic acid/12% methanol solution for 30 minutes, transferred to a Whatman 3 MM paper (Whatman, Maidstone, Kent, England), covered with plastic film, and dried under vacuum on a gel dryer (Slab Dryer Model 483; Bio-Rad) at 80°C for 1 hour. The gel was then unwrapped and autoradiographed by overnight exposure to a Kodak X-ray film.

6. **Tests of Spleen Function.** Apart from examining each patient for palpable enlargement, spleen function was also assessed by two methods: a) Percent of circulating red cells that have "pits" on their membrane, and b) scintigraphy.

6/a. **Pitted Red Cell Counts.** This test depends on the surveillance function of the spleen in removing damaged, old red cells from the circulation both by phagocytosis and filtration. The "pits" on the RBCs are actually vacuoles that develop with age. In the normal individual, there should be less than 3.5% of these in circulation (15,16). The methodology involves fixing a freshly collected drop of blood in 2% glutaraldehyde. A wet preparation of this mixture is made and viewed under a direct interference phase-contrast microscope (Zeiss Axiophot; Oberkochen, Germany) with Normarski optics (17). At least 500 RBCs are examined at x 1,000 and the percentage of cells with one or more surface indentations ("pits" or "pocks") is calculated.

6/b. **^{99m}Tc -Labelled Tin or Denatured RBC Scintigraphy.** The patients whose splenic reticulo-endothelial function was assessed with scintigraphy had labelled tin and/or denatured RBC uptake studies. An appropriate dose of ^{99m}Tc -labelled tin colloid (Amersham) was injected IV. The denatured RBC test was not done on the same patient until at least 2 days had elapsed. For the latter, a partial *in vitro* technique (18,19) was used. Briefly, the patients were injected with cold stannous pyrophosphate (Amersham). Twenty minutes later, about 2 ml blood was withdrawn and centrifuged. Appropriate quantities of $^{99m}\text{TcO}_4^-$ were added to the RBCs and incubated for 10 minutes. The ^{99m}Tc -RBCs were heat-denatured in a water-bath at a constant temperature of 49.5°C for 15 minutes. These were then re-injected into the patient. Radionuclide images of the posterior, left lateral, and anterior views of the splenic area were obtained after 30 minutes. The scintigraphic image was compared in intensity to that of the liver and was graded as normal or partial visualisation or non-visualisation.

REFERENCES

1. Dimovski, A.J.: Factors affecting the fetal hemoglobin levels in patients with sickle cell anemia and thalassemia. Thesis, University of Limburg, Maastricht, The Netherlands, 1993.
2. Righetti, P.G., Gianazza, E., Bianchi-Bosisio, A., and Cossu, G.: Conventional isoelectric focusing and immobilized pH gradients for hemoglobin separation and identification. In: *The Hemoglobinopathies*, edited by T.H.J. Huisman, Methods in Hematology, Vol. 15, page 47, Churchill Livingstone, Edinburgh, 1986.
3. Betke, K., Marti, H.R., and Schlicht, I.: Estimation of small percentages of foetal haemoglobin. *Nature*, 184:1877, 1959.
4. Bissé, E. and Wieland, H.: High-performance liquid chromatographic separation of human haemoglobins. Simultaneous quantitation of foetal and glycated haemoglobins. *J. Chromatogr.*, 434:95, 1988.
5. Kutlar, A., Kutlar, F., Gu, L.-H., Mayson, S.M., and Huisman, T.H.J.: Fetal hemoglobin in normal adults and β -thalassemia heterozygotes. *Hum. Genet.*, 85:106, 1990.
6. Shelton, J.B., Shelton, J. R., and Schroeder, W.A.: High performance liquid chromatographic separation of globin chains on a large-pore C₄ column. *J. Liq. Chromatogr.*, 7:1969, 1984.
7. Kutlar, F., Kutlar, A., and Huisman, T.H.J.: Separation of normal and abnormal hemoglobin chains by reversed-phase high-performance liquid chromatography. *J. Chromatogr.*, 357:147, 1986.
8. Alter, B.P., Goff, S.C., Efremov, G.D., Gravely, M.E., and Huisman, T.H.J.: Globin chain electrophoresis: A new approach to the determination of the G_{γ}/A_{γ} ratio in foetal haemoglobin and to studies of globin synthesis. *Br. J. Haematol.*, 44:527, 1980.
9. Schroeder, W.A. and Huisman, T.H.J.: *The Chromatography of Hemoglobin, Clinical and Biochemical Analysis*, Vol.9, Marcel Dekker, Inc., New York, 1980.
10. Poncz, M., Solowiejczyk, D., Harpel, B., Mory, Y., Schwartz, E., and Surrey, S.: Construction of human gene libraries from small amounts of peripheral blood: Analysis of β -like globin genes. *Hemoglobin*, 6:27, 1983.
11. Matteucci, M.D. and Carruthers, M.H.: Synthesis of deoxyoligonucleotides on a polymer support. *BioTechnology*, 24:92, 1992.
12. Saiki, R.K., Scharf, S., Faloona, F., Mullis, K.B., Horn, G.T., Erlich, H.A., and Arnheim, N.: Enzymatic amplification of β -globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science*, 230:1350, 1985.
13. Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A.: Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science*, 239:487, 1988.
14. Wood, W.G., Gitschia, J., Lasky, L.A., and Lawn, R.N.: Base composition independent hybridization in tetramethylammonium chloride. A method for oligonucleotide screening of highly complex gene libraries. *Proc. Natl. Acad. Sci. USA*, 82:1585, 1985.
15. Sanger, F., Nicklen, S., and Coulson, A.R.: DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*, 74:5463, 1977.

16. Koyama, S., Kihira, H., Aoki, S., and Ohnishi, H.: Postsplenectomy vacuole: A new erythrocyte inclusion body. *Mie Med. J.*, 11:425, 1962.
17. Kent, G., Minnick, O.T., Volini, F.I., and Orfei, E.: Autophagic vacuoles in human red cells. *Am. J. Pathol.*, 48:831, 1966.
18. Pearson, H.A., Johnson, D., Smith, K.A., and Touloukian, R.J.: The born-again spleen: Return of reticulo-endothelial function after splenectomy for trauma. *N. Engl. J. Med.*, 298:1389, 1978.
19. Armas, R.R., Thakur, M.L., and Gottschalk, A.: A simple method of spleen imaging with Tc-99m labelled erythrocytes. *Radiology*, 132:215, 1979.
20. Owunwanne, A., Halkar, R., Al-Rashied, A., Aboobacker, K.C., and Abdel-Dayem, H.: Radionuclide imaging of the spleen with heat denatured technetium-99m RBC when the splenic reticuloendothelial system seems impaired. *J. Nuclear Med.*, 29:320, 1988.

CHAPTER 3

STUDIES AMONG NIGERIANS

Chapter 3a	Persistent Gross Splenomegaly in Nigerian Patients With Sickle Cell Anaemia: Relationship to Malaria and Clinical and Laboratory Features Associated With Persistent Gross Splenomegaly in Nigerian Children With Sickle Cell Anaemia	55
Chapter 3b	Spleen in Sickle Cell Anaemia: Comparative Studies of Nigerian and U.S. Patients	65
Chapter 3c	Frequency of the α -Thalassaemia-2 Gene Among Nigerian SS Patients and Its Influence on Malaria Antibody Titres	77
Chapter 3d	Haplotypes in SS Patients From Nigeria; Characterisation of One Atypical β^S Haplotype No. 19 (Benin) Associated With Elevated Hb F and High G_γ Levels	85

CHAPTER 3a

**PERSISTENT GROSS SPLENOMEGALY IN NIGERIAN PATIENTS
WITH SICKLE CELL ANAEMIA: RELATIONSHIP TO MALARIA**

A.D. Adekile, O.O. Adeodu, A.A. Jeje, and W.O. Odesanmi

(Adapted from: *Ann. Trop. Paediatr.*, 8:103-107, 1988)

AND

**CLINICAL AND LABORATORY FEATURES ASSOCIATED WITH PERSISTENT
GROSS SPLENOMEGALY IN NIGERIAN CHILDREN WITH SICKLE CELL ANAEMIA**

O.O. Adeodu and A.D. Adekile

(Adapted from: *Acta Paediatr. Scand.*, 79:686-690, 1990)

ABSTRACT

Although malaria is believed to contribute to splenomegaly among SS patients in the tropics, it is not known whether there are other factors associated with persistent gross splenomegaly (PGS), *i.e.* spleens of ≥ 10 cm below the costal margin in children ≥ 8 years. Among 139 steady state SS patients aged 6 months to 15 years, 47 (33.8%) had splenomegaly and 15 (10.8%) met the criteria for PGS. Serum IgM, hepatic histopathology, and response to prolonged proguanil treatment were investigated in the latter and compared to age- and sex-matched SS patients without splenomegaly, and AA controls. The mean $\pm$ SD serum IgM levels were 249.7 ± 75 , 157.6 ± 57.3 , and 146.2 ± 48.9 mg/dl, respectively; the differences are statistically significant ($p < 0.05$). Hepatic histopathology, in 11 of the patients, showed moderate to severe sinusoidal dilatation and portal lymphocytic infiltration, while three had evidence of new fibrous tissue formation and basement membrane degeneration. Of the 13 patients that were followed on daily proguanil therapy, 10 showed remarkable reduction in splenic size within 6 months; the mean splenic size regressed significantly ($p < 0.01$) from 13.8 ± 3.2 to 5.6 ± 5.3 cm over the period. PGS was also associated with frequent anaemic crisis, hepatomegaly, and digital clubbing. These studies show that PGS, in SS patients, is similar to tropical splenomegaly syndrome (TSS),

which is believed to be a hypersensitivity state to malaria, while a few demonstrate pre-cirrhotic hepatic pathology.

INTRODUCTION

Splenomegaly is a common finding in patients with SS. In the infant and young child, it is due to the combined effects of extramedullary haemopoiesis, congestion and, occasionally, sequestration. However, recurrent infarction leads to fibrosis and autosplenectomy that usually occurs by the age of 8-10 years, after which less than 10% of American SS patients have palpable spleens (1-3). Conversely, however, splenomegaly tends to persist to a much older age in African patients. Esan (4) has reported a spleen rate of 15% in adult Nigerian SS patients. This persistence is believed to be related to the effect of malaria (5,6).

There is ample evidence that the β^S gene, especially in the heterozygote, confers appreciable protection against malaria and its complications (7-9). While Vandepitte (10) reported lower parasite rates in SS patients in comparison to trait and normal individuals, several investigators have reported severe and frequent clinical malaria in their homozygous patients. Indeed, malaria is probably the most significant precipitant of crisis in those patients living in the tropics (5,11).

The other disease in the tropics which causes massive splenomegaly whose aetiology is related to malaria is TSS (12-15), which is characterised by the following: a) Chronic gross splenomegaly in the absence of a definable aetiology; b) lymphocytosis, mainly in the hepatic sinusoids, spleen, bone marrow, and occasionally in the peripheral blood; c) increased levels of serum IgM and very high malarial antibody titres; d) complete remission on prolonged administration of antimalarial drugs, *e.g.* proguanil. The present study was designed to investigate the relationship of PGS in SS patients (*i.e.* aged ≥ 8 years with splenic size ≥ 10 cm) to TSS.

MATERIALS AND METHODS

Patients. This study was carried out over 2 years. The patients were consecutive homozygous SS patients seen in the first 6 months of the study. The diagnosis of SS was based on solubility studies and Hb electrophoresis. Each patient had to be in steady state, *i.e.* not in crisis or any other acute or chronic illness that could be responsible for splenomegaly. Informed consent was obtained from all the parents of the patients and controls in the study.

Past clinical records were reviewed and a detailed physical examination was carried out. Features and complications of the disease, *e.g.* skull bossing, gnathopathy, peripheral lymphadenopathy, leg ulcers, digital clubbing, etc. were noted. The spleen was measured along its longest axis from the left costal margin and the liver along the right nipple line.

At the end of 6 months, the percentage of the total patients seen who had a consistently palpable spleen while in a steady state was calculated. The patients with gross splenomegaly were screened for the exclusion of the following: Tuberculosis (Mantoux test and chest X-ray), parasitic infections (blood films, stool and urine for ova and parasites), and primary blood dyscrasias (peripheral blood examination).

Serum IgM Levels. Serum samples were obtained from venous blood of the patients with PGS and from two control groups. The first control group consisted of age- and sex-matched SS patients without splenomegaly. The second were age- and sex-matched normal Hb AA individuals who had no acute or chronic illness and no palpable spleen. IgM concentrations were obtained with the single radial immunodiffusion method using Tri-partigen plates (Behringwerke).

Proguanil Therapy. The patients with PGS were started on oral proguanil, 100 mg daily. Drug compliance was advised and monthly appointments were given for replenishing drug supplies. The initial splenic size was recorded in each patient and measurements were taken at the end of the 3rd and 6th months of proguanil therapy. At these visits, the patient had to be in a steady state for the spleen size to be recorded. If he/she had any acute illness, this was treated appropriately and the patient given another appointment for measurement of the spleen size at least 2 weeks after the resolution of such an illness. The mean spleen size at the commencement of proguanil was compared to the value at 6 months.

Hepatic Histopathology. In those patients with gross splenomegaly liver biopsies were performed prior to or just before commencing proguanil therapy. This was to rule out liver cirrhosis, which is a known complication of SS. Immediate post-mortem liver biopsies were successfully obtained in four SS patients, without splenomegaly or evidence of chronic hepatic disease, who died in the emergency room during the period of the study. These served as controls.

Data Analysis. Results are expressed as mean $\pm$ SD. Significance of the difference between mean values was determined using Student's t test or analysis of variance (ANOVA). Values of $p < 0.05$ were taken to imply statistical significance.

RESULTS

A total of 139 patients (aged 6 months to 15 years, with a mean age of 7.1 ± 4.2 years) were recruited into the study. Of these, 47 (33.8%) had palpable spleens at the initial visit. Among this group the mean age was 7.0 ± 0.6 years which was not significantly ($p > 0.05$) different from the mean age of those without splenomegaly (7.2 ± 0.7 years). The range of spleen size was 4-21 cm, with a mean of 11.7 ± 1.1 cm. Of those aged ≥ 8 years, 19 (13.7%) had palpable spleens. Fifteen (10.8%) had splenic size ≥ 10 cm. These included 10 females and five males, with a mean age of 11.3 ± 2.7 years. Fig. 3a/1 is a histogram of the distribution of splenic sizes in the study population.

FIG. 3a/1. Histogram showing the distribution of spleen sizes in the study.

All the patients with PGS complained of a left-sided abdominal mass and/or dull pain on recruitment into the study. All had felt the mass for periods ranging from 9 months to over 2 years. All had experienced a gradual increase, while none thought it had been decreasing in size. In 10 patients, it was the presence of the mass that prompted the parents to bring the child to the hospital.

All the patients had used antimalarials on previous occasions, but none was on regular malaria chemoprophylaxis before first presentation and recruitment into the study. None showed evidence of gross malnutrition.

Past Medical History. Table 3a/1 shows the frequency of crises, hospital admissions and blood transfusions in SS patients with PGS and the control group over a 2-year period. The former suffered more from anaemic crises and less from vaso-occlusive crises than those without splenomegaly ($\chi^2 = 4.03$, $p < 0.05$) and $\chi^2 = 5.47$, $p < 0.05$, respectively).

Associated Clinical Features. There was no difference in the weight and height of both groups. Hepatomegaly (in 93.3%), generalized peripheral lymphadenopathy (in 33.3%), and gnathopathy (in 40%) were more frequent among the PGS group than the controls (60, 0, and 0%, respectively). Digital clubbing was present in 33.3% of the former and in 10% of the latter.

The mean PCV, WBC, and platelet counts were significantly higher in the control group, while the serum indirect bilirubin and globulin were significantly higher in the PGS group ($p < 0.01$).

Serum IgM Levels. Serum IgM levels were obtained in nine (60%) of the patients with PGS. Nine age- and sex-matched SS patients without splenomegaly and nine other age- and sex-matched AA individuals served as controls. Table 3a/2 summarizes the results. The mean IgM values were 249.7 ± 75.0 mg/dl in SS patients with PGS, 157.6 ± 57.3 mg/dl in SS patients without splenomegaly, and 146.2 ± 48.9 mg/dl in the AA individuals. Analysis of variance (ANOVA) showed that the differences between the mean values were statistically significant ($p < 0.01$).

Proguanil Therapy. Of the 15 patients originally commenced on proguanil, one defaulted from follow-up and one died at home during the study. The splenic size at the commencement of the study ranged from 10-21 cm (mean 13.8 ± 3.2 cm). At 3 months, the range was 2-19 cm (mean 8.9 ± 4.3 cm), and 0-19 cm (mean 5.6 ± 5.3 cm) at 6 months. The difference between the mean at commencement and at 6 months is statistically significant ($p < 0.05$).

Ten (67%) patients had a brisk reduction of more than 5 cm in splenic size during the period of study; these were termed "responders". In the remaining three (23%), the reduction was less than 5 cm, with two having a reduction of only 2 cm; they were termed "poor responders" (Fig. 3a/2). The mean age of the latter was 13.0 ± 2.1 years, while that of the responders was 10.8 ± 2.8 years; the difference was not significant ($p > 0.05$). However all the poor responders had had enlarged spleens for more than 2 years.

TABLE 3a/1. Frequency of Crises, Admissions, and Blood Transfusions in SS Patients With PGS and SS Patients Without Splenomegaly

		SS Patients With PCG (n = 15)		Control SS Patients Without Splenomegaly (n = 10)	
		Episodes	n % of Total	Episodes	n % of Total
Anaemic crises		28	14 93.3	5	5 50 <0.05
Vaso-occlusive crises		12	11 73.3	30	10 100 <0.05
Admissions		14	8 53.3	8	6 60 NS
Blood transfusion		10	8 53.3	5	5 50 NS

TABLE 3a/2. IgM Titres (mg/100 ml) in SS Patients With PGS and in Controls

No.	Age (years)	SS With PGS Spleen Size (cm)	IgM	SS Controls IgM	AA Controls IgM
1	15	13	396	192	192
2	8	12	266	288	112
3	15	20	232	142	230
4	11	21	266	124	164
5	8	10	169	150	96
6	13	12	262	130	110
7	15	11	262	96	110
8	12	12	120	284	192
9	14	12	274	112	110
x SD	12.3±2.8	13.7±7.2	249.7±75.0	157.6±57.3	146.2±48.9

FIG. 3a/2. Patterns of response to proguanil therapy.

Hepatic Histopathology. Liver biopsy was successfully performed in 11 (73%) of the patients with PGS. All showed sinusoidal dilatation with a moderate degree of lymphocytic infiltration in eight, but severe in three. Two of the latter had the largest spleens in the study. Portal lymphocytic infiltration and Kupffer cell hyperplasia with pigment inclusions were evident in all but one. Six (54.5%) patients' slides showed areas of necrosis, while some degree of focal fibrosis was seen in four (36.4%). Generally, however, the hepatic architecture was preserved in all. Though cirrhosis was not evident in any of the patients, a reticulin stain of the slides of the poor responders showed evidence of new fibrous tissue formation and basement membrane degeneration. The control slides showed evidence of mild lymphocytic infiltration of the portal tracts and sinusoids with sinusoidal dilatation. The liver architecture was preserved and no areas of necrosis or degeneration were found.

DISCUSSION

The present study has demonstrated some similarity between TSS and PGS in SS patients. The major similarity, apart from the degree of splenomegaly, is in the uniform response to proguanil. The only three who responded poorly had enlarged spleens for more than 2 years, and one had evidence of calcification on plain abdominal X-ray. It would, therefore, appear that duration of splenomegaly affects response to proguanil and that children with long-standing splenomegaly probably retain their palpable spleens to adulthood.

Watson et al (16) showed that once the spleen is palpable in an SS child, it takes 6 months to 5.5 years for it to completely regress in size. We, therefore, believe that the prompt reduction in size within 6 months reflects the effects of proguanil and not just a natural progression of the disease. This view is supported by Hendrickse (17), who also reported rapid decrease of spleen size in SS patients on prolonged malaria chemoprophylaxis.

The hepatic sinusoidal dilatation and lymphocytic infiltration in our patients is again similar to that reported in TSS. However, these are common findings in SS patients generally (17,18) and were also seen in the four children who served as controls. The finding of new fibrous tissue formation on reticulin staining in those not responding to proguanil suggests that they are at risk for hepatic cirrhosis later in life, as reported in other studies (19-21).

The IgM values in the SS patients with PGS were significantly higher than in the controls, but much lower than those reported by Sagoe (860-3,000 mg/dl) for adult TSS patients (14). However, the results show that the spleen in PGS patients is actively producing immunoglobulins. We have also recently shown that splenic reticulo-endothelial function is preserved in these patients as shown by significantly lower pitted red cell counts in comparison to SS patients without splenomegaly (22).

Although the aetiology of TSS is not known, there is considerable circumstantial evidence linking it with malaria. The same seems to be true of PGS in Nigerian SS patients. There is a need for regular malaria chemoprophylaxis in SS patients in the

tropics and patients with PGS need to be followed closely because of the higher frequency of anaemic crisis, and the fact that some of them might be at risk for cirrhosis. Future studies should investigate possible correlation between spleen size and malaria antibody titres. Also the influence of genetic factors (β -globin gene haplotypes and co-existent α -thal) on splenomegaly need to be examined.

REFERENCES

1. Powars, D.R.: Natural history of sickle cell disease - the first ten years. *Semin. Hematol.*, 12:267, 1975.
2. Smith, E.W. and Conley, C.L.: Clinical features of genetic variants of sickle cell disease. *Bull. Johns Hopkins Hosp.*, 94:289, 1954.
3. Scott, R.B. and Ferguson, A.D.: Studies in sickle cell anemia. XXVII: Complications in infants and children in the United States. *Clin. Pediatr.*, 5: 403, 1966.
4. Esan, G.F.: The clinical spectrum of sickle cell anaemia in Nigerian adults. *INSERM.*, 44:43, 1982.
5. Kaine, W.N.: Sickle cell anaemia in children in Eastern Nigeria. *East Afr. Med. J.*, 59:742, 1982.
6. Bwibo, N.O. and Kasili, E.G.: Clinical aspects of sickle cell disease in Nairobi children. *Am. J. Pediatr. Hematol./Oncol.*, 4:187, 1982.
7. Allison, A.C.: Protection afforded by sickle cell trait against subtertian malaria. *Br. Med. J.*, 1:290, 1954.
8. Luzzatto, L., Nwachukwu-Jarrett, E.S., and Reddy, S.: Increased sickling of parasitized erythrocytes as mechanism of resistance against malaria in sickle cell trait. *The Lancet*, i:319, 1970.
9. Roth, E.F., Friedman, M., Ueda, Y., Tellez, I., Trager, W., and Nagel, R.L.: Sickling rates of human AS red cells infected *in vitro* with *Plasmodium falciparum* malaria. *Science*, 202:650, 1975.
10. Vandepitte, J.M.: Some remarks on the pathology of Haemoglobin S. In: *Abnormal Haemoglobins in Africa*, edited by J.H.P. Jonxis, Blackwell Scientific Publications, Oxford, 1963.
11. Konotey-Ahulu, F.I.D.: Malaria and sickle cell disease. *Br. Med. J.*, 2:710, 1971.
12. Edington, G.M.: Malaria in the tropics. *Br. Med. J.*, 1:715, 1967.
13. Watson-Williams, E.J. and Allan, N.C.: Idiopathic tropical splenomegaly syndrome in Ibadan. *Br. Med. J.*, 3:378, 1970.
14. Sagoe, A.S.: Tropical splenomegaly syndrome: Long-term proguanil therapy correlated with spleen size, serum IgM and lymphoblastic transformation. *Br. Med. J.*, 3:378, 1970.
15. Fakunle, Y.M.: Tropical splenomegaly. *Clin. Haematol.*, 10:963, 1981.
16. Watson, R.J., Lichman, H.C., and Shapiro, H.D.: Splenomegaly in sickle cell anaemia. *Am. J. Med.*, 20:196, 1956.
17. Hendrickse, R.G.: Sickle cell anaemia in Nigerian children. *Cent. Afr. J. Med.*, 6:45, 1960.

18. Brew, J. and Edington, G.M.: Haemoglobins S and C in post-mortem material in Ibadan, Nigeria. In: *Abnormal Haemoglobins in Africa*, edited by J.H.P. Jonxis, Blackwell Scientific Publications, Oxford, 1963.
19. Alli, A.F. and Lewis, E.A.: The liver in sickle cell disease - pathological aspects. *Ghana Med. J.*, 6:119, 1969.
20. Green, T.W., Conley, C.L., and Berthrong, M.: The liver in sickle cell anaemia. *Bull. Johns Hopkins Hosp.*, 92:99, 1953.
21. Rosenblatt, H.J., Eisenstein, K., and Holmes, A.W.: The liver in sickle cell anaemia: A clinical study. *Arch. Pathol.*, 90:235, 1970.
22. Adekile, A.D., Reindorf, C.A., Adeodu, O.O., Winston, J., and Dairo, B.A.: Pitted red cell counts in Nigerian SS patients. *J. Natl. Med. Assoc.*, 83:329, 1991.

CHAPTER 3b

SPLEEN IN SICKLE CELL ANEMIA:

COMPARATIVE STUDIES OF NIGERIAN AND U.S. PATIENTS

A.D. Adekile^{1,2}, K.M. McKie³, O.O. Adeodu², A.J. Sulzer⁴, J-S. Liu¹,
 V.C. McKie³, F. Kutlar¹, M. Ramachandran¹, W. Kaine⁵,
 G.I. Akenzua⁶, A.A. Okolo⁶, A.A. Asindi⁷, E.A. Obinyan⁸,
 W.N. Ogala⁹, M. Ibrahim¹⁰, and T.H.J. Huisman¹

¹ Department of Biochemistry and Molecular Biology
 Medical College of Georgia, Augusta, GA 30912-2100, USA

² Department of Paediatrics and Child Health
 Obafemi Awolowo University, Ile-Ife, Nigeria

³ Department of Pediatrics, Medical College of Georgia, Augusta, GA 30912, USA

⁴ Malaria Branch, Centers for Disease Control, Atlanta, GA 30303, USA

⁵ Institute of Child Health, University of Nigeria Teaching Hospital, Enugu, Nigeria

⁶ Department of Child Health, University of Benin Teaching Hospital, Benin, Nigeria

⁷ Department of Paediatrics
 University of Calabar Teaching Hospital, Calabar, Nigeria

⁸ Department of Paediatrics, Institute of Health
 Ahmadu Bello University, Kaduna, Nigeria

⁹ Department of Paediatrics, Ahmadu Bello University, Zaria, Nigeria

¹⁰ Department of Paediatrics
 Uthman Dan Fodio University Teaching Hospital, Sokoto, Nigeria

(Adapted from: Am. J. Hematol., 42:316-321, 1993)

ABSTRACT

Anecdotal reports have attributed persistent splenomegaly in African SS patients to the effects of malaria. However, no comparative studies of patients in malarial and non-malarial regions have been conducted, and few studies of malaria antibody titres have been reported. In the present study, age- and sex-matched Nigerian and U.S. steady-state SS patients were compared. Splenomegaly was found in 22.3% of Nigerian patients ($n = 310$), while it was found in only 8% of U.S. patients ($n = 100$) from Georgia. There was significant linear correlation between spleen size and Hb levels and with serum immunoglobulins in the Nigerian group. However, serum complement levels (C3 and C4) were not affected by spleen size. In both groups, patients with splenomegaly had fewer circulating pitted red cells than their counterparts without splenomegaly. The mean $\pm$ SE of IgG-specific malaria antibody titre among the Nigerian patients without palpable spleens was $9,386 \pm 2,036$; $9,334 \pm 2,980$ in those with spleens between 1 and 5 cm, $16,201 \pm 4,502$ in those with spleens between 6 and 10 cm, and $22,445 \pm 8,456$ in those with spleens above 10 cm. Coexistent α -thal did not influence the prevalence of splenomegaly among the Nigerian SS patients. This study provides additional evidence that malaria plays a significant role in the persistence of splenomegaly in African patients.

INTRODUCTION

The spleen usually becomes palpably enlarged early in the life of an SS patient (1,2). The reasons for this include extramedullary hemopoiesis, congestion, and occasionally sequestration. However, the recurrent vasoocclusion and infarction that characterise the disease cause a regression of the spleen as the child gets older. By the age of 10 years, the spleen has undergone autosplenectomy and is no longer palpable in most American patients. However, it has been well documented (3,4) that the spleen remains palpable in much older patients living in tropical Africa. In a previous study (3), we showed that splenomegaly occurred in one-third of Nigerian SS patients aged between 10 and 16 years, while Esan (4) found splenomegaly in 15% of adult Nigerian patients. Although the reason for this difference is not quite clear, some reports have suggested that malaria is responsible for the persistent enlargement of the spleen in African patients, and effective antimalarial chemoprophylaxis has been shown to reduce the prevalence of splenomegaly (3).

The present study was designed to analyse the occurrence of splenomegaly in SS patients in a malaria-endemic region (Nigeria) and a non-malaria region (Georgia, United States). Splenic function was investigated using pitted red cell counts. Among the Nigerian SS patients, the relationship between splenomegaly and the following parameters were investigated: Haematological data, coexistent α -thal, serum immunoglobulins (IgG, IgM, and IgA), and complement (C3 and C4) and malaria antibody titres.

MATERIALS AND METHODS

Nigerian Patients. Patients were recruited from sickle cell clinics in seven centres in Nigeria, four in the south (Ile-Ife, Benin, Enugu, and Calabar) and three in the north (Zaria, Kaduna, and Sokoto). These clinics were selected to reflect the different ethnic groups in the population (Yoruba, Hausa/Fulani, and Ibo are the major tribes, but over 40 others are represented in the study).

The patients were randomly selected in the clinics during the period of collection (January, 1991). Blood samples were also obtained from a few patients who were hospitalised at the time of the study. Informed consent was obtained from each of the patients or from their parents. A questionnaire was completed to reflect the current health status of each patient.

A control group of normal individuals, with either Hb AA or Hb AS, was obtained using relatives of the SS patients or children visiting the welfare clinics of the different centers.

U.S. Patients. These were known SS patients attending the pediatric clinic of the Comprehensive Sickle Cell Center at the Medical College of Georgia, Augusta, GA, or one of its satellite clinics and were matched for age and sex with the Nigerian patients. The same questionnaire as was used for the Nigerian subjects was completed for each individual.

Physical Examination. Each individual in the study was examined by a physician, and abnormal findings were documented. The current state of health of the patient was noted (i.e. was the patient in steady state, or did he/she have a crisis or any acute or chronic illness?). The spleen and liver sizes were measured along the midclavicular line below the costal margin, using a nonelastic tape measure. These measurements were the averages of figures obtained by two observers. Patients with any chronic disease not related to SS were excluded from the study.

Blood Sampling. At least 5 ml of blood was collected in vacutainers containing an anticoagulant (EDTA). Another 5 ml of blood was obtained from Southern Nigerian and U.S. patients into vacutainers without anticoagulant. About 0.1 ml of fresh blood was mixed with 1 ml 2% glutaraldehyde buffer, pH 7.2, for pitted red cell counts.

The EDTA samples were stored at 4°C until transported to Augusta, within 1 month of collection. Sera were obtained from the coagulated samples and stored frozen until analysed.

Laboratory Methods. CBCs were obtained with an electronic counter (Sysmex K-1000). Hb analyses included IEF (5) to confirm the diagnosis of SS and to determine the Hb genotype of the controls along with cation exchange HPLC (6,7) for the quantitation of Hb F. DNA was extracted from leukocytes by the method of Poncz et al (8) and α -globin gene mapping was done on Nigerian SS patients with methods routinely used in our laboratory (9,10).

Counting of circulating pitted red cells was done with a direct interference phase-contrast microscope with Nomarski optics (11) (Zeiss Axiophot). A wet preparation of the mixture of blood in glutaraldehyde was made, and at least 500 consecutive red cells were examined at X 1,000. The percentage of cells with one or more surface indentations, commonly referred to as "pits" or "pocks", was calculated (12,13).

Serum immunoglobulins A, M, and G, and complement C3 and C4 were determined by nephelometry (14,15). The IgG- and IgM-specific malaria antibody titres were determined by indirect fluorescent assay (16).

Data Analysis. Data are presented as mean $\pm$ SD, and Student's *t* test was used to test the significance of differences between mean values. The χ^2 test and Pearson's correlation coefficient (*r* values) were computed where necessary. All statistical analyses were done with Statgraphics computer software.

RESULTS

Patients and Controls. A total of 310 Nigerian SS patients (156 males and 154 females) were studied. Their ages ranged from 1 to 25 years, with a mean of 9.7 ± 0.3 years. One hundred seventeen age- and sex-matched controls were also available: 91 were AS (aged 3-26 years, mean 8.7 ± 1.8 years) and 26 were AA (aged 5-18 years, mean 10.0 ± 1.8 years). One hundred U.S. SS patients (52 males and 48 females) were recruited into the study. Their ages ranged from 1 to 18 years, with a mean of 9.0 ± 0.5 years.

Splenomegaly Among the SS Patients and Controls. Sixty-nine of 310 Nigerian SS patients (22.3%) had palpable spleens. The spleen size ranged from 1 to 17 cm, with a mean of 6.5 ± 0.5 cm. Of the patients aged 5 years and under, 20.3% had splenomegaly; between 6 and 10 years, the figure was 25.7%, between age 11 and 15 years, 23.7%, and after the age of 15 years, 17.9%. No significant difference in the mean ages of those with (9.3 ± 0.6 years) or without (9.8 ± 0.4 years) splenic enlargement was observed. However, of the 69 with splenomegaly, 41 (59.4%) were females, while 28 (40.6%) were males ($\chi^2 = 3.5$, $P = 0.06$).

Among patients who were in steady state, 38 of 187 (20.3%) had palpable spleens of 2 cm or more. Among the 25 patients with acute malaria, five (20%) had splenomegaly, while four of 23 (17.4%) with acute bacterial infections (pneumonia, osteomyelitis, and septicemia) and four of 15 (26.7%) with vasoocclusive crisis had palpable spleens. All the laboratory data generated in this study on these acutely ill patients were not significantly different from those of patients in steady state, except that the patients with malaria and septicemia had more severe anemia.

When the data were analysed on a regional basis, patients from Southern Nigeria (58 out of 182; 31.9%) were more likely to have splenomegaly than those from the north (11 of 128; 8.6%), but no association with particular ethnic groups was demonstrated. Five of the 91 AS individuals from Nigeria (5.5%) had palpable

spleens of 2-8 cm; their age range was 4-12 years. Among the 26 AA individuals, two (7.7%) had palpable spleens of 2 and 6 cm; aged 2 and 11 years, respectively.

Eight (8%) of the 100 U.S. SS patients had palpable spleens of 2-5 cm; their age range was from 3 to 18 years, with a mean of 7.4 ± 1.8 years, but only two were aged over 10 years. There was an equal sex distribution (four each).

Haematology. Table 3b/1 lists the haematological data for Nigerian and U.S. SS patients with and without splenomegaly. Data for the Nigerian controls are also included. While the data for the Nigerian patients should be interpreted with caution because of the effects of storage prior to analysis, the results were not significantly different from those of U.S. patients. Striking decreases in RBC counts and Hb were observed in the Nigerian patients with increasing splenomegaly. This was not seen among the U.S. patients, probably because the splenomegaly was of a lesser degree.

TABLE 3b/1. Mean Haematological Data According to Spleen Size

Spleen (cm)	n	RBC $10^{12}/l$	Hb g/dl	MCH pg	Hb F %
<u>Nigerian SS Patients</u>					
All	302 ^a	2.81	7.6	27.1	9.3
0	238	2.92	7.8	27.3	9.0
1-5	30	2.82	7.5	26.4	11.3
6-10	25	2.15	6.3	30.4	11.8
>10	9	1.74	5.4	30.6	11.2
<u>U.S. SS Patients</u>					
All	100	2.77	8.0	29.4	13.7
0	92	2.75	7.9	29.6	8.5
1-5	8	3.09	8.6	27.7	14.3
<u>Nigerian AS Controls</u>					
All	81	4.34	11.7	26.9	1.5
<u>Nigerian AA Controls</u>					
All	26	4.23	11.7	27.6	0.6

^a Data for some patients were unsatisfactory; values for PCV, MCV, and MCHC are omitted because the effect of storage on the samples made these measurements unreliable.

α -Thal. α -Globin gene mapping was done on 259 Nigerian SS patients, 54 of whom had splenomegaly. Among the latter, 33.3% were heterozygous for an α -thal-2 deletion, and 9.3% were homozygous. Of the 205 SS patients who did not have

splenomegaly, the figures were 39 and 7.8%, respectively. There were no significant differences in these proportions ($\chi^2 = 0.6$, $P = 0.7$). Details of our findings with respect to α -thal in this study are being reported elsewhere (17).

Pitted RBC Counts. Table 3b/2 lists the pitted RBC counts for Nigerian and U.S. patients. The results were similar in both groups. Patients with splenomegaly had lower counts. This was most striking among the Nigerian group, with a correlation coefficient of pit counts with splenic size of -0.3 ($P < 0.005$).

TABLE 3b/2. Mean $\pm$ SD Pitted RBC Counts in Nigerian and U.S. SS Patients

Spleen (cm)	Nigerian		U.S.	
	n	% of Cells	n	% of Cells
All	94	14.6 $\pm$ 1.1	78	14.0 $\pm$ 1.2
0	64	17.7 $\pm$ 1.4	70	14.4 $\pm$ 1.3
1-5	11	11.9 $\pm$ 2.2	8	10.2 $\pm$ 1.8
6-10	13	10.4 $\pm$ 2.0	--	----
>10	6	7.5 $\pm$ 1.6	--	----

Immunoglobulin and Complement Levels (C3 and C4). Table 3b/3 lists the serum immunoglobulin and complement data for Nigerian and U.S. SS patients, arranged according to spleen size. The values of IgM, IgG, and IgA were significantly higher ($P < 0.001$) in the Nigerian patients than in the U.S. patients, but differences in the complement values were not significant ($P > 0.05$). The immunoglobulin values in the Nigerian patients increased with increasing spleen size. The correlation coefficients of splenic size with immunoglobulin values in the Nigerian patients were 0.4 ($P < 0.001$) for IgM, 0.5 ($P < 0.001$) for IgG, and 0.3 ($P < 0.005$) for IgA. Although the mean IgM value was higher in the U.S. patients with splenomegaly than in those without, the difference was not significant ($P > 0.05$).

Malaria Antibody Titres. These titres were determined in 102 Nigerian patients (Table 3b/3). There was a significant increase in the titre with an increase in the size of the spleen. The correlation coefficient of spleen size with IgG-specific antibody was 0.3 ($P < 0.005$) and with IgM-specific antibody 0.1 ($P > 0.05$); that between serum IgG and IgG-specific malaria antibody titre was 0.4 ($P < 0.001$), and that between serum IgM and the IgM-specific malaria antibody titre was 0.5 ($P < 0.001$). The malaria antibody titres for 53 AS individuals and 18 AA individuals are also included in Table 3b/3.

DISCUSSION

There is increasing interest in the role of the spleen in immune response in SS patients because of the predisposition to bacterial infections, which is the main cause

TABLE 3b/3. Mean $\pm$ SD Serum Ig, Complement, and Malaria Antibody Titres According to Spleen Size

Spleen (cm)	n	IgG mg/dl	IgA mg/dl	IgM mg/dl	C3 mg/dl	C4 mg/dl	n	IgG Anti- body Dil. Factor	IgM Anti- body Dil. Factor
<u>Nigerian SS Patients and Controls</u>									
All	140	2314.8 $\pm$ 100.1	255.9 $\pm$ 14.6	562.9 $\pm$ 147.7	74.0 $\pm$ 3.2	28.4 $\pm$ 1.4	102	9937 $\pm$ 1409	74 $\pm$ 29
0	99	1887.0 $\pm$ 112.2	218.0 $\pm$ 16.1	283.0 $\pm$ 33.2	73.1 $\pm$ 4.0	28.7 $\pm$ 4.0	48	9386 $\pm$ 2036	26 $\pm$ 5
1-5	15	2401.3 $\pm$ 193.7	245.0 $\pm$ 37.2	407.6 $\pm$ 107.7	78.8 $\pm$ 9.0	30.5 $\pm$ 3.5		9334 $\pm$ 2980	291 $\pm$ 96
6-10	17	3048.8 $\pm$ 254.8	317.4 $\pm$ 37.4	885.1 $\pm$ 208.7	74.2 $\pm$ 9.5	26.6 $\pm$ 2.8	22	16201 $\pm$ 4502	51 $\pm$ 27
>10	9	2934.0 $\pm$ 417.6	326.3 $\pm$ 67.5	1809.3 $\pm$ 1366.7	69.8 $\pm$ 6.0	27.7 $\pm$ 5.5	9	22443 $\pm$ 8456	54 $\pm$ 18
AS Adults		n.d.	n.d.	n.d.	n.d.	n.d.	53	16478 $\pm$ 2583	77 $\pm$ 22
AA Adults		n.d.	n.d.	n.d.	n.d.	n.d.	18	24949 $\pm$ 14766	26 $\pm$ 0.5
<u>U.S. SS Patients</u>									
All	59	1391.1 $\pm$ 56.6	223.4 $\pm$ 12.7	173.5 $\pm$ 11.2	61.9 $\pm$ 1.2	26.8 $\pm$ 0.9		n.d.	n.d.
0	51	1419.7 $\pm$ 64.1	226.0 $\pm$ 14.0	166.4 $\pm$ 11.6	61.8 $\pm$ 1.3	26.5 $\pm$ 1.0		n.d.	n.d.
1-5	8	1208.6 $\pm$ 58.0	207.0 $\pm$ 30.5	218.6 $\pm$ 33.8	62.8 $\pm$ 2.7	28.5 $\pm$ 1.3		n.d.	n.d.

n.d. = not determined.

of death in children with this disease (1,2,18). The present study has again confirmed previous findings that splenomegaly is much more common in African SS patients than in SS patients living in Europe or the United States. Surprisingly, however, the prevalence of splenomegaly among SS patients in Northern Nigeria was similar to the figure among U.S. patients in this study. The reason for this is not clear.

Nigeria, a West African country, is hyperendemic for malaria. Malaria parasite rate is at its highest in preschool children, with rates of 90% or more in this group in the maximum transmission period, which is during the wet season (19). The parasite rate remains high in children between the ages of 5 and 10 years; a maximum of 90% is present in the 5-7 year age group, but it falls to 50 to 75% in the 8-10 year age group, 20-50% in the 11-15 year age group, and 10-30% in adults (19). The present study was conducted during the dry season, when the transmission rate of malaria is higher in the rain forest belt of the south than the savanna grassland of the north. This may account for the difference in prevalence of splenomegaly between northern and Southern Nigerian patients.

While it has been previously suggested that persistent splenomegaly in African SS patients is due, to some extent, to the effects of recurrent malaria (3), the present study is the first to compare splenic function between SS patients from a malaria zone and those living in a malaria-free zone. The study demonstrates relative hypersplenism among SS patients with splenomegaly as previously reported (20). Reticuloendothelial function appears to be enhanced, as shown by the lower red cell pit counts in the patients with splenomegaly. This would indicate that, although functional asplenia has been described in SS patients with splenomegaly (21-23), reticuloendothelial function is not uniformly compromised in such patients.

The spleen increases greatly in size during malaria infection, mainly due to an initial proliferation of splenic T cells, on which recovery from malaria is dependent, followed by hypertrophy of the B cell system (24,25). Indeed, Greenwood and Vick (26) postulated that malaria parasites produce a mitogen that activates B cells. This is responsible for elevated levels of the three classes of immunoglobulins that have been reported in residents of malaria endemic regions all over the world. On the other hand, malaria tends to reduce levels of complement, especially C3.

Previous investigators have shown that serum immunoglobulin levels in SS patients are often significantly higher than those in normal controls (27-32). However, the present study has demonstrated that all the immunoglobulin fractions (IgG, IgA, and IgM) were significantly higher in Nigerian than in U.S. SS patients, while the differences in complement levels were not significant. However, the values are higher in SS patients with gross splenomegaly. Complement values, on the other hand, are not influenced by splenic size. Serum immunoglobulins and complement were not studied among Northern Nigeria patients, in whom the prevalence of splenomegaly turned out to be similar to that in U.S. patients.

Further evidence of the role of malaria in splenomegaly among Nigerian SS patients is provided by the data on malaria antibody titres. Most of the specific malaria antibodies were in the IgG fraction, as expected, since IgM antibodies are of

importance only in the first two years of life (33). Although there was a wide variation in the values, the data demonstrate more intense antigenic stimulation in AA individuals, as was previously reported (34). However, a marked increase in the IgG-specific antibody titre was noted among SS patients with large spleens, while a correlation was observed between the levels of serum IgG and IgG-specific malaria antibody titre. This finding is contrary to that reported by Molineaux and Gramiccia (35), who postulated that the elevated serum immunoglobulin levels in SS patients are not related to malaria antibody levels.

Acknowledgements. This work was supported by Fogarty International research grant IF05TW04415-01 awarded to ADA. The authors thank the resident doctors in the different hospitals who assisted with the collection of samples. Dr. D.E. Bockman of the Department of Anatomy, Medical College of Georgia, made available the phase-contrast microscope used for pit counts. We thank Ms. Joyce Oliver, Ms. Martha Harrington, and Mr. Emmitt L.D. Walker, III, for technical assistance; Mrs. Alice L. Reese for administrative and logistics support, and Mrs. Felicia O. Okeagu for secretarial assistance. ADA also thanks Beecham Research International for additional support.

REFERENCES

1. Powars, D.R.: Natural history of sickle cell disease - The first 10 years. *Semin. Hematol.*, 12:267, 1975.
2. Scott, R.B. and Ferguson, A.D.: Studies in sickle cell anemia. XXXVII: Complications in infants and children in the United States. *Clin. Pediatr.*, 5:403, 1966.
3. Adekile, A.D., Adeodu, O.O., Jefe, A.A., and Odesanmi, W.A.: Persistent splenomegaly in sickle cell anemia: Relationship to malaria. *Ann. Trop. Pediatr.*, 8:103, 1988.
4. Esan, G.J.F.: The clinical spectrum of sickle cell disease in Nigerian adults. *INSERM*, 44:43, 1966.
5. Righetti, P.G., Gianazza, E., Bianchi-Bosisio, A., and Cossu, G.: Conventional isoelectric focusing and immobilized pH gradients for hemoglobin separation and identification. In *The Hemoglobinopathies*, edited by T.H.J. Huisman, *Methods in Hematology*, Vol. 15, page 47, Churchill Livingstone, Edinburgh, 1986.
6. Bissé, E. and Wieland, H.: High-performance liquid chromatographic separation of human haemoglobins. Simultaneous quantitation of foetal and glycated haemoglobins. *J. Chromatogr.*, 434:95, 1988.
7. Kutlar, A., Kutlar, F., Gu, L-G., Mayson, S.M., and Huisman, T.H.J.: Fetal hemoglobin in normal adults and β -thalassemia. *Hum. Genet.*, 85:106, 1990.
8. Poncz, M., Solowiejczyk, D., Harpel, B., Mory, Y., Schwartz, E., and Surrey, S.: Construction of human gene libraries from small amounts of peripheral blood: Analysis of β -like globin genes. *Hemoglobin*, 6:27, 1982.
9. Felice, A.E., Ozdonmez, R., Headlee, M.E., and Huisman, T.H.J.: Organization of α chain genes among Hb G-Philadelphia heterozygotes in association with Hb S, β -thalassemia and α -thalassemia-2. *Biochem. Genet.*, 20:689, 1982.

10. Felice, A.E., Cleek, M.P., McKie, K., McKie, V., and Huisman, T.H.J.: The rare α -thalassemia-1 of Blacks is a $\zeta\alpha$ -thalassemia-1 associated with deletion of all α - and ζ -globin genes. *Blood*, 63:1253, 1984.
11. Koyama, S., Kihira, H., Aoki, S., and Ohnishi, H.: Postsplenectomy vacuole: New erythrocyte inclusion body. *Mie Med. J. Jpn.*, 11:425, 1962.
12. Kent, G., Minick, O.T., Volini, Fl., and Orfei, E.: Autophagic vacuoles in human red cells. *Am. J. Pathol.*, 48:831, 1966.
13. Pearson, H.A., Johnston, D., Smith, K.A., and Touloukian, R.J.: The born-again spleen: Return of splenic reticulo-endothelial function after splenectomy for trauma. *N. Engl. J. Med.*, 298:1389, 1978.
14. Killingsworth, L.M. and Savory, J.: Nephelometric studies of the precipitin reaction: A model system for specific protein measurement. *Clin. Chem.*, 19:403, 1973.
15. Buffone, G.J.: Immunonephelometric and turbidometric measurement of specific plasma proteins. In *Manual of Clinical Immunology*, 2nd edition, edited by N.R. Rose and H. Friedman, Am. Soc. Microbiol., Washington, D.C., 1980.
16. Sulzer, A.J. and Wilson, M.: The fluorescent antibody test for malaria. *CRC Critical Reviews, Clin. Lab. Sci.*, 2:601, 1971.
17. Adekile, A.D., Liu, J-S., Sulzer, A.J., and Huisman, T.H.J.: Frequency of the α -thalassemia-2 gene among Nigerian SS patients and its influence on malaria antibody titers. *Hemoglobin*, 17:73, 1993.
18. Pearson, H.A.: Sickle cell anemia and severe infection due to encapsulated bacteria. *J. Infect. Dis.*, 136(Suppl.):43a25, 1977.
19. Salako, L.A.: Malaria in Nigeria. In *Proceedings of the Conference on Malaria in Africa*, edited by A.A. Buck, page 131, American Institute of Biological Sciences on behalf of the U.S. Agency for International Development, Washington, D.C., 1986.
20. Adeodu, O.O. and Adekile, A.D.: Clinical and laboratory observations on sickle cell patients with gross splenomegaly. *Acta Paediatr. Scand.*, 79:687, 1990.
21. Pearson, H.A., Spencer, R.P., and Cornelius, E.A.: Functional asplenia in sickle cell anemia. *N. Engl. J. Med.*, 281:923, 1969.
22. Adekile, A.D., Adeodu, O.O., Reindorf, C.A., Johnson, W., and Dairo, B.A.: Pitted red cell counts in Nigerian children with sickle cell anemia: Relationship to splenic size and age. *J. Natl. Med. Assoc.*, 83:329, 1991.
23. Fatunde, O.J. and Scott, R.B.: Pitted red cell counts in sickle cell disease. *Am. J. Pediatr. Hematol. Oncol.*, 8:329, 1985.
24. Roberts, D.W. and Weidanz, W.P.: Splenomegaly, enhanced phagocytosis and anemia are thymus-dependent responses to malaria. *Infect. Immunol.*, 20:728, 1978.
25. Edington, G.M.: Malaria in the tropics. *Br. Med. J.*, 1:715, 1967.
26. Greenwood, B.M. and Vick, R.M.: Evidence for a malaria mitogen in human malaria. *Nature*, 257:592, 1975.
27. Gavrilis, P., Rothenberg, S.P., and Guy, R.: Correlation of low serum IgM levels with absence of functional splenic tissue in sickle cell disease syndromes. *Am. J. Med.*, 57:542, 1974.
28. De Ceular, K., Forbes, M., Maude, G.H., Pagliucca, A., and Serjeant, G.R.: Complement and immunoglobulin levels in early childhood in homozygous sickle cell disease. *J. Clin. Lab. Immunol.*, 21:37, 1986.

29. Al-Awamy, B., Niazi, G.A., Al-Mouzan, M.I., Al-Nahdi, M., Naeem, M.A., and Sumer, T.: Serum immunoglobulin and complement levels in patients with sickle cell anemia from eastern province of Saudi Arabia. *Trop. Geogr. Med.*, 40:13, 1988.
30. Adeodu, O.O. and Adekile, A.D.: Serum immunoglobulins A and M in sickle cell patients from Ile-Ife, Nigeria. *E. Afr. Med. J.*, 66:631, 1989.
31. Cohen, S. and Deans, J.A.: Specific acquired immunity in experimental malaria. In *Principles and Practice of Malariology*, edited by W.H. Wernsdorfer and I.A. McGregor, page 515, Churchill Livingstone, Edinburgh, 1988.
32. McGregor, I.A. and Wilson, R.J.M.: Specific immunity: Acquired in man. In *Principles and Practice of Malariology*, edited by W.H. Wernsdorfer and I.A. McGregor, page 559, Churchill Livingstone, Edinburgh, 1988.
33. Molineaux, L., Fleming, A.F., Cornille-Brögger, R., Kagan, I., and Storey, J.: Abnormal hemoglobins in the Sudan savanna of Nigeria. III. Malaria, immunoglobulins and antimalarial antibodies in sickle cell disease. *Ann. Trop. Med. Parasitol.*, 73:301, 1979.
34. Cornille-Brögger, R., Fleming, A.F., Kagan, I., Matsushima, T., and Molineaux, L.: Abnormal hemoglobins in the Sudan savanna of Nigeria. II. Immunological responses to malaria in normals and subjects with sickle cell trait. *Ann. Trop. Med. Parasitol.*, 73:173, 1979.
35. Molineaux, L. and Gramiccia, G.: *The Garki Project: Research on the Epidemiology and Control of Malaria in the Sudan Savanna of West Africa*, World Health Organization, Geneva, 1988.

CHAPTER 3c

**FREQUENCY OF THE α -THALASSAEMIA-2 GENE AMONG NIGERIAN SS PATIENTS
AND ITS INFLUENCE ON MALARIA ANTIBODY TITRES****A.D. Adekile¹, J-C. Liu¹, A.J. Sulzer², and T.H.J. Huisman¹**¹ Department of Biochemistry and Molecular Biology
Medical College of Georgia, Augusta, GA 30912-2100, USA² Malaria Branch, Centers for Disease Control, Atlanta, GA 30333, USA**(Adapted from: Hemoglobin, 17:73-79, 1993)****ABSTRACT**

The frequency of the α -thal-2 gene was determined in 284 Nigerian SS patients and 62 age- and sex-matched AS and AA controls, using standard gene mapping techniques. The study subjects were drawn from different cities in the country. IgG malaria-specific antibody and anti-RESA antibody titres were determined in those patients from the rain forest belt of Southern Nigeria, with an indirect fluorescent technique and the influence of co-existent α -thal-2 trait on the values obtained was investigated. The frequency of the α -(3.7 kb) deletion among the SS patients was 0.258 and 0.24 in the controls, while heterozygosity for the α -globin gene triplication was 0.009. The α -thal-2 frequency was higher in the rain forest belt of the south and central parts of the country, with the Ibos in the southeast having the highest frequency (0.33) and the Fulanis of the extreme north, the lowest (0.04). Almost all the individuals studied had positive IgG malaria antibody titres ($\geq 1:64$); however, 8.3% of SS, 14% AS, and 19.2% AA had very high titres ($\geq 1:65,536$). One hundred and thirty-six (19.1%) of the SS, seven (14%) of the AS, and two (8.3%) of the AA had positive anti-RESA antibody titres. However, when these titres were analysed for the presence of the α -thal-2 gene, 23.2% of patients with four functional genes, 17.5% with three genes, and 0% of those with two genes, had positive titres. The study confirms the low IgG malaria antibody titres that have been described in AS and SS individuals who tend to have low parasite loads. It also appears that anti-RESA antibodies may play a role in the immunity afforded by SS disease and α -thal against malaria.

INTRODUCTION

The distribution of α - and β -thal in different parts of the world closely corresponds to the distribution of current or recent malaria endemicity. As with some structural Hb polymorphic variants (S, C, and E), the thalassaemias offer relative protection against malaria infection (1-5), although the molecular mechanisms responsible are not quite clear. However, in β^S heterozygotes or homozygotes, parasite-enhanced and hypoxia-induced sickling leads to preferential destruction of parasitised RBCs (3). On the other hand, there is evidence of compromised growth of malaria parasites in Hb H-containing RBCs, but not in patients with less severe forms of α -thal (4). It has also been postulated that parasitised α -thal RBCs are more susceptible to oxidant damage, and infection may produce sufficient oxidative stress to alter intracellular metabolism, leading to premature death of the parasite (5,6). The impact of these protective mechanisms on malaria antibody sero-activity in SS patients with or without a co-existent α -thal has not been widely reported.

Nigeria is situated in West Africa between latitudes 5° and 13° to the north of the equator, and occupies a land area of $\sim 924,000 \text{ km}^2$ with a population of about 100 million. The people of the country are derived from Sudanese and Bantu Negro stocks with a few Hamitic types in the extreme north (7). There are over 250 ethnic groups, but the majority are Yorubas in the southwest, Ibos in the southeast, and Hausa/Fulani in the north. The country is hyperendemic for malaria with transmission occurring throughout the year (8); Hb S is the most common haemoglobinopathy with a frequency of 0.25 (9,10).

We have recently conducted a study of β^S haplotypes and α -thal in a large number of Nigerian SS patients. Here we report the α -thal-2 frequencies for different centres in Nigeria and provide preliminary data on how its concomitant presence influences malaria antibody titres in SS patients.

MATERIALS AND METHODS

The subjects were steady state SS patients attending sickle cell clinics in eight centres in the country (Fig. 3c/1). Their age- and sex-matched AS and AA siblings served as controls. Whole blood was obtained by venepuncture into two vacutainers (one plain and one with EDTA as anticoagulant) from each individual. Informed consent was obtained from the parents of all individuals in the study. Serum was obtained by centrifugation of the coagulated sample and stored frozen at -20°C , while the EDTA sample was stored at 4°C . The samples were transported (the blood samples on wet ice and the sera in dry ice) to Augusta, GA, USA within a month of collection by the first author.

Haematological data were obtained on arrival in Augusta, GA, USA using an automated cell counter (Sysmex, Kobe, Japan). All samples were analysed by IEF (11) and by cation exchange HPLC for the quantitation of Hb F and Hb A₂ (12,13). Hb F was isolated by DEAE-cellulose chromatography and its γ chain composition was determined by reversed phase HPLC (14).

FIG. 3c/1. Map of Nigeria showing the eight cities involved in the study and the frequency of the α -thal-2 (3.7 kb) deletion at each centre.

DNA was isolated from leukocytes with the method of Poncz et al (15). The presence of α -thal-2 trait was determined by gene mapping methodology routinely in use in our laboratory (16,17). The distribution of the trait was examined for the different centres and tribes in the study.

Only patients from the rain forest belt of Southern Nigeria (Ile-Ife, Benin, and Enugu) were involved in malaria antibody titre studies. Serum malaria-specific IgG and anti-RESA antibody titres were determined by indirect fluorescent assays (18) at the Malaria Branch of the Centers for Disease Control in Atlanta, GA, USA. The latter is a neo-antigen with a molecular mass of 155,000 daltons and its immuno-dominant domain is a tandem repeat of eight amino acids (19). It is released from dense granules within *P. falciparum* merozoites after invasion of the red cell and transported to the erythrocyte cytoskeleton via a series of organelles and membranes (19-21). Previous work has shown that anti-RESA antibodies are the most likely to correlate with protective immunity, and they show an inverse correlation with parasite density (19,20).

RESULTS

Two hundred and eighty-four SS patients and 62 controls were studied. Only one type of α -thal was detected; namely $-\alpha(-3.7 \text{ kb})$, while a few chromosomes with the corresponding triplication, i.e. $\alpha\alpha\alpha$ (anti 3.7 kb), were observed. The frequency of the α -thal-2 (-3.7 kb) gene was 0.258 in the SS patients and 0.24 in the control group. Heterozygosity for the α -globin gene triplication was observed in five SS patients [$f(\alpha\alpha\alpha) = 0.009$]

The limited haematological data for the SS patients showed an increase in Hb level and red cell count, and a decrease in MCH and MCV values, with a decrease in the number of functional α -globin genes (Table 3c/1). The mean age of the SS patients with a concomitant α -thal-2 homozygosity in the present study was significantly higher than in those with four intact genes and those with three genes (α -thal-2 heterozygotes).

Differences were noted in the distribution of the $-\alpha(-3.7 \text{ kb})$ chromosome among patients attending different sickle cell centres. The frequency of α -thal-2 was high in the rain forest belt of the south and central parts of the country, and considerably less in the three northern centres which lie in the grassland/arid zone (Fig. 3c/1). When the frequencies among the major tribes were examined, the Yoruba and Hausa had similar values of 0.25 and 0.21, respectively, but the Ibo had a significantly higher value of 0.33 ($\chi^2 = 29.08$, $p < 0.001$), while α -thal-2 was rare among the Fulani [$f(-\alpha) = 0.04$].

Of the 153 SS patients who had serum malaria-specific IgG antibody titres determined, 139 (90.8%) had positive titres ($\geq 1:64$), while 50 of 52 (96.2%) AS and all (100%) of the 26 AA were positive (Table 3c/2). When individuals with positive titres were subdivided according to the levels of observed titres, 12 of 139 (8.3%) SS, seven of 50 (14%) AS, and five of 26 (19.2%) AA subjects had very elevated

TABLE 3c/1. Haematological Data for 220 SS Patients With a Homozygosity for the β^S Haplotype #19 (Benin)^a

# of α Genes	n	Age years	Hb g/dl	RBC $10^{12}/l$	MCV fl	MCH pg	Hb A ₂ ^b %	Hb F ^b %
4	123	9.5 $\pm$ 5.6	7.4 $\pm$ 1.3	2.59 $\pm$ 0.52	108.7 $\pm$ 10.7	28.8 $\pm$ 13.3	2.8 $\pm$ 0.9	9.5 $\pm$ 5.4
3	92	9.5 $\pm$ 5.9	7.8 $\pm$ 1.4	2.97 $\pm$ 0.61	98.6 $\pm$ 7.3	26.3 $\pm$ 3.0	3.1 $\pm$ 0.9	9.1 $\pm$ 6.0
2	15	12.8 $\pm$ 6.8	8.2 $\pm$ 1.6	3.76 $\pm$ 0.89	85.7 $\pm$ 14.3	21.3 $\pm$ 1.6	3.6 $\pm$ 1.7	7.6 $\pm$ 5.4

^a Average $\pm$ SD values.

^b By cation exchange HPLC.

TABLE 3c/2. IgG and Anti-RESA Malaria Antibody Titres in Patients With or Without an α -Thal-2 Deletion (figures in parentheses are percentages)

<u>Patients and Controls</u>		<u>SS Patients and α Gene Status</u>					
Antibody	Titre	SS n = 153	AS n = 52	AA n = 26	$\alpha\alpha/\alpha\alpha$ n = 64	$-\alpha/\alpha\alpha$ n = 47	$-\alpha/-\alpha$ n = 9
IgG	<1:64	14 (9.2)	2 (3.8)	0	3 (4.7)	7 (14.9)	1 (10.1)
	1:64-1:256	27 (17.6)	3 (5.8)	3 (11.5)	13 (20.3)	9 (19.1)	1 (10.1)
	1:1024-1:16384	100 (65.4)	40 (76.9)	18 (69.2)	43 (67.2)	28 (59.6)	7 (77.8)
	>1:65536	12 (7.8)	7 (12.5)	5 (19.2)	5 (7.8)	3 (6.4)	0
		n = 136	n = 50	n = 24	n = 56	n = 40	n = 8
Anti-RESA	<1:64	110 (80.9)	43 (86.0)	22 (91.7)	43 (76.8)	33 (82.5)	8 (100.0)
	>1:64	26 (19.1)	7 (14.0)	2 (8.3)	13 (23.2)	7 (17.5)	0

($\geq 1:65,536$) titres. The differences in these rates were also not significant ($\chi^2 = 3.0$, $p = 0.2$).

Twenty-six of 136 (19.1%) SS patients, seven of 50 (14%) AS, and two of 24 (8.3%) AA had positive anti-RESA antibody titres (Table 3c/2). Although the differences in this distribution were not statistically significant ($\chi^2 = 2.04$, $p = 0.36$), interestingly, it is a direct reversal of the distribution of the same subjects with severely elevated IgG antibody titres (see above).

The α -globin gene status was determined for 120 of the SS patients who had malaria-specific IgG antibody titres. In this group, 64 had four functional α -globin genes, while 47 were heterozygous (three genes), and nine were homozygous (two genes) for the α -thal-2 deletion. In these three groups, 5.3, 85.1, and 97.9%, respectively, had positive malaria-specific IgG antibody titres (Table 3c/2). However, when those who had positive titres were subdivided according to the levels of the titres, 91.8, 92.5, and 100%, respectively, had mild (1:64 to 1:256) to moderate (1:1024 to 1:16,384) elevations of IgG titres as opposed to those with severe elevation ($\geq 1:65,536$). When anti-RESA antibody titres were analysed among SS patients according to the presence of α -thal-2, 23.2% of patients with four α genes, 17.5% with three α genes, and 0% with two α genes, had positive titres ($\geq 1:64$) (Table 3c/2).

DISCUSSION

The frequency of α -thal-2 in the present study compares favourably with those reported by others for the Nigerian population (22,23) and is also about the same as observed for American Blacks (24-26). However, this is the first report of α -globin gene triplications among Nigerians. It is interesting that the frequencies of α -thal-2 trait were different among the various Nigerian ethnic groups. How much this reflects true genetic differences is unknown. However, unlike the other ethnic groups in the study, the Fulanis, with the lowest frequency (0.04), are considered Saharan/Hamitic in origin (7,27) and share some physiognomic features with North African Arabs, in whom the frequency of α -thal-2 has been reported to be 0.04-0.18 (28).

The increased levels of Hb and RBC in SS patients with homozygosity or heterozygosity for the α -thal-2 gene are likely the result of decreased haemolysis observed in those with an α chain deficiency (29) because of a decreased MCHC. In addition, these children are less prone to complications such as leg ulcers, renal pathology, and strokes (24,25,30). Co-existent α -thal-2 is also associated with increased survival in SS children (31) as borne out in the present study, although an association with a milder clinical course, based on the frequency of crises, hospitalisations, and complications, could not be established.

While the pattern of malaria-specific IgG antibody titres did not show significant differences in the subgroups, the lower percentage of positivity in the SS and AS groups could be a reflection of lower parasite densities in these two groups as

reported by previous studies (32). There is also an indication that SS patients with co-existent α -thal may have less intense sero-reactivity.

The pattern of anti-RESA antibody titres suggests that a cellular mechanism involving the interaction of *P. falciparum* merozoites and receptors on the red cell membrane should be considered as part of the mechanisms of immunity afforded by the β^S gene against severe malaria infection. It is interesting that SS patients with an α chain deficiency had the lowest titres of anti-RESA antibodies. Although these results are preliminary and not definitive, they provide an indication for further studies to define the role of anti-RESA antibodies in the immunity afforded by SS and α -thal against malaria. Moreover, Luzzi et al (33,34) have recently reported increased *in vitro* binding of antibodies to neo-antigens at the surface of infected α -thal trait erythrocytes, suggesting enhanced immune recognition.

Acknowledgements. The authors thank Drs. W. Kaine, G.I. Akenzua, A.A. Okolo, A.A. Asindi, E.A. Obinyan, W.L. Ogala, and I. Watila for their assistance in collecting the blood samples and for permitting us to include their patients in this study. The study was supported in part by a Fogarty International research fellowship Award No. IF05TW004414-01 (to ADA) and by the United States Public Health Services research grant HLB-41544 (to THJH).

REFERENCES

1. Livingstone, F.B.: *Frequencies of Hemoglobin Variants*, Oxford University Press, New York, 1985.
2. Weatherall, D.J.: *Ann. Trop. Med. Parasitol.*, 81:539, 1989.
3. Nagel, R.L. and Roth, E.F.: *Blood*, 74:1213, 1989.
4. Ifediba, T.C., Stern, A., Ibrahim, A., and Rieder, R.F.: *Blood*, 65:452, 1985.
5. Friedman, M.J.: *Nature*, 280:245, 1979.
6. Allison, A.C. and Eugui, E.M.: *The Lancet*, 2:1431, 1982.
7. *Nigeria - Background Notes*, edited by J. Adams, U.S. Department of State, Bureau of Information, Office of Public Information, Washington, D.C., 1987.
8. Salako, L.A., Ajayi, F.O., Sowunmi, A., and Walker, O.: *Ann. Trop. Med. Parasitol.*, 84:435, 1990.
9. Walters, J.H. and Lehmann, H.: *Trans. Roy. Soc. Trop. Med. Hyg.*, 50:204, 1956.
10. Jelliffe, D.B. and Humphreys, J.: *Br. Med. J.*, 1:405, 1952.
11. Righetti, P.G., Gianazza, E., Bianchi-Bosisio, A., and Cossu, G.: In: *The Hemoglobinopathies*, edited by T.H.J. Huisman, *Methods in Hematology*, Vol. 15, page 47, Churchill Livingstone, Edinburgh, 1986.
12. Bissé, E. and Wieland, H.: *J. Chromatogr.*, 434:95, 1988.
13. Schroeder, W.A. and Huisman, T.H.J.: *The Chromatography of Hemoglobin, Clinical and Biochemical Analysis*, Vol. 9, Marcel Dekker, Inc., 1980.
14. Kutlar, A., Kutlar, F., Gu, L-G., Mayson, S.M., and Huisman, T.H.J.: *Hum. Genet.*, 85:106, 1990.

15. Poncz, M., Solowiejczyk, D., Harpel, B., Mory, Y., Schwartz, E., and Surrey, S.: *Hemoglobin*, 6:27, 1982.
16. Felice, A.E., Ozdonmez, R., Headlee, M.E., and Huisman, T.H.J.: *Biochem. Genet.*, 20:689, 1982.
17. Felice, A.E., Cleek, M.P., McKie, K., McKie, V., and Huisman, T.H.J.: *Blood*, 63:1253, 1984.
18. Sulzer, A.J. and Wilson, M.: *CRC Critical Reviews, Clin. Lab. Sci.*, 2:601, 1971.
19. Coppel, R.L., Cowman, A.F., Anders, R.F., Bianco, A.E., Saint, R.B., Lingel-Bach, K.R., Kemp, D.J., and Brown, G.V.: *Nature*, 310:789, 1984.
20. Ravetch, J.V., Young, J., and Poste, G., *BioTechnology*, 3:729, 1985.
21. Culvenor, J.G., Day, K.P., and Anders, R.F.: *Infect. Immunol.*, 59:1183, 1991.
22. Falusi, A.G., Esan, G.J.F., Ayyub, H., and Higgs, D.R.: *Eur. J. Haematol.*, 38:370, 1987.
23. Pagnier, J., Dunda-Belkhodja, O., Zohoun, I., Teyssier, J., Baya, H., Jaeger, G., Nagel, R.L., and Labie, D.: *Hum. Genet.*, 68:318, 1984.
24. Embury, S.H., Dozy, A.M., Miller, J., Davis, J.R., Kleman, K.M., Preisler, E., Vichinsky, W.N., Lande, W.N., Lubin, B.H., Kan, Y.W., and Mentzer, W.C.: *N. Engl. J. Med.*, 306:270, 1982.
25. Embury, S.H.: *Ann. N.Y. Acad. Sci.*, 565:213, 1989.
26. Higgs, D.R., Aldridge, B.E., Lamb, J., Clegg, J.B., Weatherall, D.J., Hayes, R.J., Grandison, Y., Lowrie, Y., Mason, K.P., and Serjeant, G.R.: *N. Engl. J. Med.*, 306:1441, 1982.
27. Adekile, A.D.: *Hemoglobin*, 16:105, 1992.
28. Higgs, D.R., Vickers, M.A., Wilkie, A.O.M., Pretorius, I-M., Jarman, A.P., and Weatherall, D.J.: *Blood*, 73:1081, 1989.
29. Noguchi, C.T., Dover, G.J., Rodgers, G.P., Serjeant, G.R., Antonarakis, S.E., Anagnou, N.P., Higgs, D.R., Weatherall, D.J., and Schechter, A.N.: *J. Clin. Invest.*, 75:1632, 1985.
30. Mears, J.G., Lachman, H.M., Labie, D., and Nagel, R.L.: *Blood*, 62:286, 1983.
31. Steinberg, M.H., Rosenstock, W., Coleman, M.B., Adams, J.G., Platica, O., Cedeno, M., Rieder, R.F., Wilson, J.T., Milner, P., and West, S.I.: *Blood*, 63:1353, 1984.
32. Molineaux, L., Fleming, A.F., Cornille-Brögger, R., Kagan, I., and Storey, J.: *Ann. Trop. Med. Parasitol.*, 73:301, 1979.
33. Luzzl, G.A., Merry, A.H., Newbold, C.I., Marsh, K., and Pasvol, G.: *Immunol. Lett.*, 30:233, 1991.
34. Luzzl, G.A., Merry, A.H., Newbold, C.I., Marsh, K., Pasvol, G., and Weatherall, D.J.: *J. Exp. Med.*, 173:785, 1991.

CHAPTER 3d

**HAPLOTYPES IN SS PATIENTS FROM NIGERIA;
CHARACTERISATION OF ONE ATYPICAL β^S HAPLOTYPE NO. 19 (BENIN)
ASSOCIATED WITH ELEVATED Hb F AND HIGH G_γ LEVELS**

**A.D. Adekile^{1,2}, M.N. Kitundu¹, L-H Gu¹, K.D. Lanclos¹,
O.O. Adeodu², and T.H.J. Huisman¹**

¹ Department of Biochemistry and Molecular Biology
Medical College of Georgia, Augusta, GA 30912-2100, USA

² Department of Pediatrics and Child Health
Obafemi Awolowo University, Ile-Ife, Nigeria

(Adapted from: *Ann. Hematol.*, 65:41-45, 1992)

SUMMARY

We have determined the haplotypes of 669 β^S and 109 β^A chromosomes from numerous members of 297 Nigerian families of various ethnic backgrounds. Among the β^S chromosomes, haplotype #19 was detected in 93%, haplotype #17 in 3.4%, and haplotype #20 in 1%, while 2.4% represented atypical haplotypes. As many as 60.6% of the β^A chromosomes exhibited haplotype #19 mutations, 8.2% had haplotype #3, and 1.8% had haplotype #20. Two siblings with elevated Hb F and G_γ levels were heterozygous for a β^S chromosome with haplotype #19 and a second chromosome with a hybrid haplotype (termed #19B). In this hybrid chromosome, haplotype #3-like LCR HS-2 sequences are in juxtaposition to those of the 5' flanking region of the G_γ promoter of a β^S chromosome with haplotype #19. The presence of this hybrid chromosome is associated with high G_γ values in individuals with both SS and AS; it closely resembles another hybrid β^S chromosome, termed #19A, observed in a previously reported Turkish SS patient who was homozygous for this chromosome and had high Hb F and G_γ values. In both instances, it is hypothesised that the haplotype #3-like sequences of the LCR HS-2 contain genetic determinants that can combine with factors produced during hematopoietic stress, resulting in increased γ -globin gene expression.

INTRODUCTION

Four major β^S haplotypes have been described in different parts of Africa, and these are referred to as haplotypes #3 (Senegal), #17 (Cameroon), #19 (Benin), and #20 [Bantu or Central African Republic (CAR)] (1,19,25). A fifth haplotype (#31, or Saudi Arabia/India) is not found in Africans. These haplotypes are associated with varying degrees of clinical severity; the disease is milder among SS patients with haplotypes #3 and #31, probably because they have higher levels of Hb F and G_γ chains.

Nigeria is situated in West Africa between latitudes 5° and 13° to the north of the equator, and occupies a land area of about 924,000 km² with a population of about 100 million. It shares borders with the Gulf of Guinea in the south, the Benin Republic in the west, the Cameroon and Chad Republics in the east, and the Niger Republic in the north. Nigerians are derived from Sudanese and Bantu Negro stocks, with a few Hamitic types in the extreme north. There are over 250 distinct ethnic groups in Nigeria, but the three largest groups are the Yoruba in the southwest, Hausa/Fulani in the north, and Ibo in the southeast (27,28).

Hb S is the most common haemoglobinopathy among Nigerians. About 25% of the population has AS and the frequency of the homozygous state is about 3% (10,29). No detailed studies of β^S haplotypes and Hb F levels have been carried out among Nigerians. The only available study is that of the *Hpa* I polymorphism (6), which does not distinguish between haplotypes #19, #20, and #17. The present study was therefore carried out with the aim of determining the distribution of the five major haplotypes of the β^S gene cluster among different ethnic groups in the country and to correlate this information with the Hb F and G_γ levels and the haematological data of the SS patients.

PATIENTS AND METHODS

The patients attended clinics in the following centres; Ile-Ife, Benin, Enugu, and Calabar in the south, and Zaria, Kaduna, Sokoto, and Maiduguri in the north. Whole blood was obtained by venipuncture into vacutainers with EDTA as anticoagulant and stored at 4°C . The containers were transported to Augusta, GA, USA, on ice within a month of collection. Informed consent was obtained.

Haematological data were obtained on arrival in Augusta using an automated cell counter. All samples were analysed by IEF (23) and by cation exchange HPLC (2,13) for the quantitation of Hb F and Hb A₂. Hb F was isolated by DEAE-cellulose chromatography (24) and its γ chain composition was determined by reversed phase HPLC (12,26).

DNA was isolated from leukocytes with the method of Poncz et al (22). Segments of ~1350 bp of both the G_γ - and A_γ -globin genes were amplified from genomic DNA with specific G_γ and A_γ 5' direct oligonucleotide primers and a common 3' reverse primer (15). Aliquots of these amplified DNA samples were blotted onto nylon membranes and hybridised to specific 5' end-labelled oligonucleotide probes. Details

of the methodology have been reported previously (4,15,21). Characterisation of chromosomes with haplotype #17 (Cameroon) was based on the absence of mutations specific for haplotypes #3, #19, #20, and #31, and on the presence of the A_{γ}^T chain detected by cation exchange HPLC (12,26). A few SS patients who were homozygous for haplotype #19 but had unusually high Hb F or G_{γ} levels were characterised further by a study of the HS-2 of the β -globin gene LCR (5,7,20). Two characteristic mutations were examined by dot-blot hybridisation (T→G at -10924 and A→G at -10905); in these two positions chromosomes with haplotype #19 have a G, while chromosomes with haplotype #3 have a T and an A, respectively (20).

RESULTS

Three hundred and thirty-five SS patients (168 male and 167 female) from 297 families were recruited into the study. Most were in steady state at the time of blood collection. Their ages ranged from 1 to 43 years, with a mean $\pm$ SD of 9.8 ± 6.1 years. Thirty normal AA (16 male and 14 female, mean age 10.1 ± 6.8 years) and 120 AS relatives (39 male and 81 female; mean age 25.3 ± 15.6 years) served as controls. There were 38 different ethnic groups, but most subjects were Yoruba, Ibo, or Hausa (115, 53, and 60 persons, respectively).

Haplotyping was done in 290 SS patients, 89 AS, and 10 AA individuals, representing 669 β^S and 109 β^A chromosomes. Table 3d/1 shows the distribution of the haplotypes. Most prevalent was haplotype #19, while 2.4% β^S chromosomes and 29.4% β^A chromosomes had atypical haplotypes. This table also gives the haplotype homozygosity and compound heterozygosity pattern in the SS, AS, and AA individuals. Two hundred and forty-nine (85.9%) SS patients, 30 (33.7%) AS, and three (30%) AA individuals were homozygous for haplotype #19.

The 23 chromosomes with haplotype #17 came from 20 families, which were distributed among the various ethnic groups (four families were Yoruba, five Hausa, and seven Ibo, while four belonged to minority tribes located in southeast Nigeria). The seven β^S chromosomes carrying haplotype #20-specific mutations came from five families (three Hausa and one each of Margi and Kanuri). The two β^A chromosomes with haplotype #20 were seen in a Yoruba family. The single β^S chromosome with haplotype #3 was from a Hausa patient. However, the nine β^A chromosomes with this haplotype were widely distributed in the country.

Mean values of some haematological data for the different haplotype combinations are shown in Table 3d/2; age and sex distributions of the data for haplotype #19 homozygotes are included. There were no significant differences between the sexes, except after the age of 15 years, when the mean RBC and Hb values were higher among males. Fig. 3d/1 shows the distribution histograms of the Hb F and G_{γ} levels in SS patients with a homozygosity for haplotype #19. The correlation coefficient (r value) between Hb F and G_{γ} was 0.5, $p < 0.001$. There was a poor correlation of either Hb F or G_{γ} with age (r values of 0.1, $p > 0.05$ and 0.04, $p > 0.05$, respectively). The mean value of Hb F among females ($9.5 \pm 5.5\%$) was not significantly different

($p > 0.05$) from that of $9.2 \pm 6.1\%$ in males. The same was the case for the G_γ values.

TABLE 3d/1. Haplotypes of β^S and β^A Chromosomes

Haplotype	n	β^S Chromosomes %	n	β^A Chromosomes %
#19	622	93.0	66	60.6
#20	7	1.0	2	1.8
#17	23	3.4	—	—
#3	1	0.2	9	8.2
Atypical	16	2.4	32	29.4
Total	669	100.0	109	100.0

Homozygosity and Compound Heterozygosity in SS, AS, and AA Individuals

Haplotype	SS		AS		AA	
	n	%	n	%	n	%
19/19	249	85.9	30	33.7	3	30.0
20/20	2	0.7	—	—	—	—
17/17	1	0.3	—	—	—	—
19/17	21	7.2	—	—	—	—
19/3	1	0.3	8	8.9	1	10.0
19/20	—	—	2	2.2	1	10.0
19/Atypical	16	5.5	49	55.1	—	—
Atypical/Atypical	—	—	—	—	5	50.0
Total	290	100.0	89	100.0	10	100.0

The large number of patients gave us the opportunity to evaluate the possible relationship of Hb F levels to clinical severity. Patients who were in steady state at the time of the study and had been free of crises and other acute illnesses in the preceding year were classified as having mild disease. Those with occasional crises who did not require hospitalisations were moderate, and those with frequent crises and hospitalisations and/or those with complications of the disease were classified as severe. Among mild patients, the mean Hb F level was $10.1 \pm 5.7\%$, for those with a moderate course it was $9.2 \pm 5.2\%$, and for those with a severe course it was $9.5 \pm 5.4\%$, showing no significant differences.

Three SS patients with an apparent homozygosity for haplotype #19 had Hb F levels of 4.4, 19, and 10.9%, and G_γ values above 60% (64.6, 72.6, and 69.7%). The first patient was not studied further because family members were not available.

TABLE 3d/2. Haematological Data $\pm$ SD For SS Patients With Different Haplotypes

Haplo- types	Age years	n	Sex	RBC $10^{12}/l$	Hb g/dl	MCH pg	Hb F %	G _γ %
19/19	1-5	32	M	2.81 $\pm$ 0.52	7.1 $\pm$ 1.3	25.7 $\pm$ 4.4	10.8 $\pm$ 6.0	42.0 $\pm$ 6.1
	1-5	36	F	2.97 $\pm$ 0.75	7.7 $\pm$ 1.4	26.4 $\pm$ 2.5	8.8 $\pm$ 5.1	43.2 $\pm$ 3.4
	6-10	41	M	2.68 $\pm$ 0.48	7.3 $\pm$ 1.1	27.5 $\pm$ 3.0	7.1 $\pm$ 5.4	38.9 $\pm$ 4.9
	6-10	38	F	2.70 $\pm$ 0.56	7.6 $\pm$ 1.2	27.9 $\pm$ 3.1	11.1 $\pm$ 5.8	42.8 $\pm$ 4.9
	11-15	33	M	2.59 $\pm$ 0.62	7.3 $\pm$ 1.4	28.5 $\pm$ 2.8	9.4 $\pm$ 5.9	41.7 $\pm$ 6.5
	11-15	32	F	2.80 $\pm$ 0.55	7.9 $\pm$ 1.2	28.4 $\pm$ 3.5	9.4 $\pm$ 5.2	42.3 $\pm$ 7.9
	>15	15	M	3.48 $\pm$ 0.83	9.4 $\pm$ 1.8	27.8 $\pm$ 3.6	7.9 $\pm$ 4.6	42.6 $\pm$ 6.5
	>15	22	F	2.65 $\pm$ 0.79	7.5 $\pm$ 1.7	29.9 $\pm$ 4.7	8.0 $\pm$ 5.2	42.7 $\pm$ 9.7
Total		249		2.77 $\pm$ 0.66	7.6 $\pm$ 1.4	27.7 $\pm$ 3.7	9.3 $\pm$ 5.4	42.1 $\pm$ 6.5
19/17		21	M/F	3.06 $\pm$ 0.59	7.7 $\pm$ 1.9	25.1 $\pm$ 4.7	7.7 $\pm$ 5.7	40.5 $\pm$ 5.6
19/20		3	M/F	2.25 $\pm$ 0.69	6.6 $\pm$ 1.6	n.a.	9.5 $\pm$ 2.9	42.2 $\pm$ 1.7
20/20		2	M	1.94 $\pm$ 0.40	5.2 $\pm$ 0.8	26.8 $\pm$ 2.3	n.a.	n.a.
19/3		1	M	n.a.	n.a.	n.a.	18.6	70.1
17/17		1	M	n.a.	n.a.	n.a.	14.9	49.2

n.a. = not available.

FIG. 3d/1. Distribution of Hb F (left) and G_{γ} (right) values among more than 200 SS patients with a homozygosity for haplotype #19.

The last two patients were siblings; the pedigree of the family is shown in Table 3d/3. The father (#748) had an Hb S heterozygosity with a low Hb S value (27.7%) because of an α -thal-2 trait; his Hb F was 2%, and the G_γ value could not be determined accurately but was estimated at <40%. The mother, with an Hb S heterozygosity and four α -globin genes (Hb S = 39.7%) also had a low Hb F (<2%), but her G_γ value was above 50 (57.1 and 61.7%; duplicate analyses). The one heterozygous son (#744) resembled the mother; he had four α -globin genes, an Hb S level of 37.5%, and an Hb F level of <2% with 76.5% G_γ . The one normal sibling (#746) had an α -thal-2 heterozygosity with <2% Hb F and low G_γ (35.9%). Haplotyping involved an evaluation of the possible presence of three mutations and one 6 bp deletion in the G_γ promoter, two mutations in the A_γ promoter, one 4 bp change in the IVS-II of the A_γ -globin gene, and two mutations in the LCR HS-2 sequence. The final data are shown in Table 3d/3; the table also lists the nts in these various positions that are specific for the β^S haplotypes #19, #3, and #20 (4,15,20). The two SS patients (#743 and #750) were homozygous for all mutations in the G_γ and A_γ promoters, and in the A_γ -IVS-II that are characteristic for β^S haplotype #19, but the nts observed in the two positions of the LCR HS-2 sequences were characteristic for haplotypes #19 and #3, indicating that one chromosome likely resulted from a 19/3 crossover event. This modified β^S haplotype #19 (termed #19B) was inherited from the mother and was also present in the brother #744; both adults had low levels of Hb F (<2%) but high G_γ values (Table 3d/3). Another interesting aspect is the observation that the β^A chromosome of the father and two of his children (#744 and #746) had changes in the G_γ and A_γ promoter sequences that are characteristic for β^S haplotype #20.

DISCUSSION

Ever since the studies of Kan and Dozy (11) there has been increasing interest in determining the β^S -globin gene cluster haplotypes in different parts of the world. This has led to increased understanding of the origin(s) of the β^S mutation and has provided an important anthropological tool for studying migrations of people of African ancestry (1,2,18,19,25). Haplotype #19 is commonly seen in Central West Africa, North Africa, the Mediterranean Basin, and in Southeastern Europe. Haplotype #3 is restricted to the Senegal area (Atlantic West Africa), while haplotype #17 has been described mainly in the Eton tribe of Cameroon and in a few Black Americans (8,16). Haplotype #20 is seen mainly in Bantus of Central, Eastern, and Southern Africa. The distribution pattern of haplotypes in the present study is not surprising; the few cases of haplotypes #3, #17, and #20 were probably introduced from other parts of Africa. However, the presence of haplotype #20 mutations in some β^S and β^A chromosomes lends credence to the hypothesis that this mutation occurred before the expansion of the Bantu, whose origin has been traced linguistically to the Nigeria/Cameroon area (18,19).

Apart from sequence variations in the G_γ and A_γ promoters in each haplotype, the levels of Hb F and G_γ chains are also distinctly different. Thus, haplotypes #19 and #20 are generally associated with low levels of Hb F and G_γ , while haplotypes #3 and #31 are associated with high levels (8,9,14). Our haplotype #19 homozygotes had Hb F levels between 0 and 28% (Fig. 3d/1), but no relationship was observed

TABLE 3d/3. Haematological and Haplotype Data for Six Members of a Nigerian Family With a Modified β^S Haplotype #19 (#19B)

Case Age	Sex-Relation-ship	Condi-tion	# of α Genes	Hb g/dl	RBC $10^{12}/l$	MCH pg	Hb S %	Hb F %	G γ %
748	M-52	Father	AS	14.1	5.87	24.0	27.2	<2.0	<40.0
749	F-45	Mother	AS	11.3	3.88	29.1	39.7	<2.0	61.7
750	F-29	Sister	SS	8.7	3.21	27.1	-	19.0	72.6
744	M-18	Brother	AS	14.6	5.18	28.2	37.5	<2.0	76.5
743	F-14	Proband	SS	8.8	3.48	25.3	-	10.9	69.7
746	F-7	Sister	AA	10.9	4.09	26.7	0	<2.0	35.9

LCR HS-2				G γ Promoter		A γ Promoter		A γ -IVS-II	
-10905 -10924 6 bp del ^a				-369	-309	-158	-657	-271	TGGG/GCAA
748 (AS)	A G	T G	+	CG	AG	CC	GT	TC	TGGG/GCAA
749 (AS)	A A	T T	-	CG	AG	CC	GT	CC	TGGG/GCAA
750 (SS)	G A	G T	-	GG	GG	CC	TT	CC	GCAA/GCAA
744 (AS)	A A	T T	+	CG	AG	CC	GT	TC	TGGG/GCAA
743 (SS)	G A	G T	-	GG	GG	CC	TT	CC	GCAA/GCAA
746 (AA)	A A	T T	+	CC	AA	CC	GG	TC	TGGG/TGGG

#19	G	G	-	G	G	C	T	C	GCAA
#3	A	T	-	C	A	T	T	C	TGGG
#20	A	T	+	C	A	C	G	T	TGGG

#19B	A	T	-	G	G	C	T	C	GCAA
#19A ^b	A	T	-	C	G	C	T	C	GCAA

^a Presence and absence is indicated by + and -, respectively.

^b From Ref. 21.

between the level of Hb F and clinical severity or hospitalisation history. It has been postulated that female SS patients tend to have higher levels of Hb F because of a genetic factor linked to the X chromosome (14,17,19). In the present study, we were unable to demonstrate significant differences in total Hb, Hb F, and red cell counts between males and females. However, Hb and red cell counts were higher in males than in females after the age of 15 years, probably as a reflection of menarche in the later.

Öner et al (20) have offered evidence that γ -globin gene expression is modulated by variations in nt sequences in the HS-2 of the LCR of the β -globin gene, and characteristic sequences were described for haplotypes #3 and #19. It was postulated that factors produced during periods of hematopoietic stress interact with genetic determinants located on the LCR HS-2 sequences of chromosome #11, and the chromosomal structure of haplotype #3 is optimal for such interactions. Furthermore, the same authors described a Turkish SS patient with a homozygosity for haplotype #19 who had abnormally high Hb F and G_γ levels. Their studies of sequence variations in the LCR HS-2 and the G_γ promoter suggested that the patient was homozygous for a β^S chromosome resulting from a crossover that placed sequences similar to the HS-2 of a β^S chromosome with haplotype #3 in juxtaposition to those of the 5' flanking region of the G_γ -globin gene of a β^S chromosome with haplotype #19 (Fig. 3d/2). A comparable crossover is observed for the two patients (#743 and #750) listed in Table 3d/3, although the crossover appears at a point 5' to that reported in the patient described by Öner et al (20) (Fig. 3d/2). The two patients were heterozygous for this hybrid β^S chromosome with haplotype #19B; their second chromosome had the common haplotype #19. This difference with the Turkish SS patient, who has a homozygosity for the hybrid β^S haplotype #19B, likely explains the lower level of Hb F in the two Nigerian patients (10.9 and 19%) compared with the 20-25% observed in the Turkish patient (20). All three patients had the marked increase in G_γ value, suggesting a major effect of the LCR HS-2 variations on the expression of the G_γ -globin gene. It is worth noting that the two AS relatives with the β^S chromosomes with haplotype #19B had low levels of Hb F, albeit with high G_γ values of 61.5 and 76.5%, respectively.

Acknowledgements. The authors thank colleagues in the various Nigerian sickle cell centres for assistance with sample collection and permission to include their patients in this study: Dr. W. Kaine, Enugu; Drs. G.I. Akenzua and A.A. Okolo, Benin; Dr. E.A. Obinyan, Kaduna; Dr. A.A. Asindi, Calabar, Drs. I. Wakila and O. Oyelami, Maiduguri; Dr. M. Ibrahim, Sokoto, and Dr. W.N. Ogala, Zaria.

REFERENCES

1. Adekile, A.D.: Anthropology of the β^S gene-flow from West Africa to North Africa, the Mediterranean, and Southern Europe. *Hemoglobin*, 16:105, 1992.
2. Antonarakis, S.E., Boehm, C.D., Serjeant, G.R., Theisen, C.E., Dover, G.J., and Kazazian, H.H., Jr.: Origin of the β^S globin gene in Blacks; the contribution of

FIG. 3d/2. Two different hybrid haplotypes in the Turkish patient M.G. (haplotype #19A) and the two Nigerian patients listed in Table 3d/3 (haplotype #19B), each with a different location for the crossover between β S chromosomes with haplotypes #3 and #19.

- recurrent mutation or gene conversion or both. *Proc. Natl. Acad. Sci. USA*, 81: 853-856, 1984.
3. Bissé, E. and Wieland, H.: High-performance liquid chromatographic separation of human haemoglobins. Simultaneous quantification of foetal and glycated haemoglobins. *J. Chromatogr.*, 494:95, 1988.
 4. Dimovski, A.J., Öner, C., Agarwal, S., Gu, Y-C., Kutlar, F., Lanclos, K.D., and Huisman, T.H.J.: Certain mutations observed in the 5' sequences of the G_γ - and A_γ -globin genes of β^S chromosomes are specific for chromosomes with major haplotypes. *Acta Haematol.*, 85:79, 1991.
 5. Enver, T., Raich, N., Ebens, A.J., Papayannopoulou, T., Constantini, R., and Stamatoyannopoulos, G.: Developmental regulation of human fetal-to-adult globin gene switching in transgenic mice. *Nature*, 344:309, 1990.
 6. Falusi, A.G. and Esan, G.J.F.: *Hpa*-1 polymorphism and the sickle gene in Nigerians. *Trop. Geogr. Med.*, 41:133, 1989.
 7. Fraser, P., Hurst, J., Collis, P., and Grosveld, F.: DNase I hypersensitive sites 1, 2 and 3 of human β -globin dominant control region direct position-dependent expression. *Nucleic Acids Res.*, 18:3503, 1990.
 8. Hattori, Y., Kutlar, F., Kutlar, A., McKie, V.C., and Huisman, T.H.J.: Haplotypes of β^S chromosomes among patients with sickle cell anemia from Georgia. *Hemoglobin*, 10:623, 1986.
 9. Hattori, Y., Kutlar, F., Mosley, C.J., Mayson, S.M., and Huisman, T.H.J.: Association of the level of G_γ chain in the fetal hemoglobin of normal adults with specific haplotypes. *Hemoglobin*, 10:185, 1986.
 10. Jelliffe, D.B. and Humphreys, J.: The sickle cell trait in western Nigeria. *Br. Med. J.*, 1:405, 1952.
 11. Kan, Y.W. and Dozy, A.M.: Polymorphism of DNA sequence adjacent to human β -globin structural gene: Relationship to sickle mutation. *Proc. Natl. Acad. Sci. USA*, 75:5631, 1978.
 12. Kutlar, F., Kutlar, A., and Huisman, T.H.J.: Separation of normal and abnormal hemoglobin chains by reversed-phase high-performance liquid chromatography. *J. Chromatogr.*, 367:147, 1986.
 13. Kutlar, A., Kutlar, F., Gu, L-G., Mayson, S.M., and Huisman, T.H.J.: Fetal hemoglobin in normal and β -thalassemia heterozygotes. *Hum. Genet.*, 85:106, 1990.
 14. Labie, D., Pagnier, C., Lapoumeroulie, C., Rouabhi, F., Dunda-Belkhodja, O., Chardin, P., Beldjord, C., Wajcman, H., Fabry, M.E., and Nagel, R.L.: Common dependency of high G_γ -globin gene expression and high Hb F levels in β -thalassemia and sickle cell anemia patients. *Proc. Natl. Acad. Sci. USA*, 82:2111, 1985.
 15. Lanclos, K.D., Öner, C., Dimovski, A.J., Gu, Y-C., and Huisman, T.H.J.: Sequence variations in the 5' flanking and IVS-II regions of the G_γ - and A_γ -globin genes of β^S chromosomes with five different haplotypes. *Blood*, 77: 2488, 1991.
 16. Lapoumeroulie, C., Dunda, O., Trabuchet, G., Mony-Lobe, M., Labie, D., Elion, J., and Krishnamoorthy, R.: A novel sickle gene of yet another origin in Africa: the Cameroon type. *Blood*, 74:63a (Suppl. 1), 1989.
 17. Morris, J., Dunn, D., Beckford, M., Grandison, Y., Mason, K., Higgs, D.R., De Celular, K., Serjeant, B.E., and Serjeant, G.R.: The haematology of homozy-

- gous sickle cell disease after the age of 40 years. *Br. J. Haematol.*, 77:388, 1991.
18. Nagel, R.L.: The origin of the Hemoglobin S gene: Clinical, genetic and anthropological consequences. *Einstein Q. J. Biol. Med.*, 2:53, 1984.
 19. Nagel, R.L. and Ranney, H.M.: Genetic epidemiology of structural mutations of the β -globin gene. *Semin. Hematol.*, 27:343, 1990.
 20. Öner, C., Dimovski, A.J., Altay, Ç., Gürgey, A., Gu, Y.-C., Huisman, T.H.J., and Lanclos, K.D.: Sequence variation in the 5' hypersensitive site-2 of the locus control region of the β^S chromosomes are associated with different levels of fetal globin in Hemoglobin S homozygotes. *Blood*, 79:813, 1992.
 21. Öner, C., Dimovski, A.J., Olivieri, N.F., Schiliro, G., Codrington, J.F., Fattoum, S., Adekile, A.D., Öner, R., Yüregir, G.T., Altay, Ç., Gürgey, A., Gupta, R.B., Jogessar, V.B., Kitundu, M.N., Loukopoulos, D., Kutlar, F., Gu, L.-H., Lanclos, K.D., and Huisman, T.H.J.: β^S Haplotypes in various world populations. *Hum. Genet.*, 89:99, 1992.
 22. Poncz, M., Solowiejczyk, D., Harpel, B., Mory, Y., Schwartz, E., and Surrey, S.: Construction of human gene libraries from small amounts of peripheral blood: Analysis of β -like globin genes. *Hemoglobin*, 6:27, 1982.
 23. Righetti, P.G., Gianazza, E., Bianchi-Bosisio, A., and Cossu, G.: Conventional isoelectric focusing and immobilized pH gradients for hemoglobin separation and identification. In: *The Hemoglobinopathies*, edited by T.H.J. Huisman, *Methods in Hematology*, Vol. 15, page 47, Churchill Livingstone, Edinburgh, 1986.
 24. Schroeder, W.A. and Huisman, T.H.J.: *The Chromatography of Hemoglobin*, Clinical and Biochemical Analysis, Vol. 9, Marcel Dekker, Inc., 1980.
 25. Schroeder, W.A. and Munger, E.S.: Sickle cell anaemia, genetic variations, and the slave trade to the United States. *J. Afr. Hist.*, 31:163, 1990.
 26. Shelton, J.B., Shelton, J.R., and Schroeder, W.A.: High-performance liquid chromatographic separation of globin chains on a large-pore C_4 column. *J. Liq. Chromatogr.*, 7:1969, 1984.
 27. Talbot, P.A.: *The Peoples of Southern Nigeria*, Vol. 4, Frank Cass, London, 1969.
 28. United States Department of State. Nigeria - background notes. In: *Bureau of Information*, edited by J. Adams, Office of Public Information, Washington, D.C., 1987.
 29. Walters, J.H. and Lehmann, H.: Distribution of the S and C haemoglobin variants in two Nigerian communities. *Trans. R. Soc. Trop. Med. Hyg.*, 50:204, 1956.

CHAPTER 4

STUDIES AMONG KUWAITIS

Chapter 4a	Influence of α -Thalassaemia Trait on Spleen Function in Sickle Cell Anaemia Patients With High Hb F	99
Chapter 4b	Morbidity, β^S Haplotype, and α -Globin Gene Patterns Among SS Patients in Kuwait	109
Chapter 4c	Molecular Characterisation of α -Thalassaemia Determinants, β -Thalassaemia Alleles, and β^S Haplotypes Among Kuwaiti Arabs	117

CHAPTER 4a

**INFLUENCE OF α -THALASSAEMIA TRAIT ON SPLEEN FUNCTION IN
SICKLE CELL ANAEMIA PATIENTS WITH HIGH HB F**

**A.D. Adekile¹, M. Tuli², M.Z. Haider¹, K. Al-Zaabi²,
S. Mohammadi², and A. Owunwanne³**

¹ Department of Paediatrics, Kuwait University, Kuwait

² Department of Nuclear Medicine, Mubarak Al-Kabeer Hospital, Kuwait

³ Nuclear Medicine, Faculty of Medicine, Kuwait University, Kuwait

(Adapted from: Am. J. Hematol., in press)

ABSTRACT

Spleen function was studied in a group of 20 Kuwaiti SS patients (aged 2 to 12 years), using ^{99m}Tc-labelled tin colloid scintigraphy. They were screened for the α -thal determinants that are prevalent in the Arabian Peninsula: - α (3.7 kb) deletion, α 2-globin gene poly A signal (AATAAA→AATAAG) mutation, and the 5' IVS-I splice junction pentanucleotide (GAGGTGAGG→GAGG) deletion with a combination of PCR and ASO hybridisation techniques. The patients were divided into three groups depending on the result of their colloid uptake. Group I consisted of seven (35%) patients with normally visualised spleens; group II, five (25%) with partial visualisation, while in group III there were eight (40%) in whom the spleen was not visualised at all. The significant distinguishing features among those in groups I and III were the MCV of 74.1 ± 5.1 and 90.1 ± 6.6 fl ($p < 0.0001$) and the MCH of 22.4 ± 2.7 and 27.5 ± 4.0 pg ($p < 0.05$), respectively. None of the patients was iron deficient, *i.e.* plasma ferritin < 12 ng/ml. The overall frequency of α -thal determinants in the study was 35%; however, the frequencies in groups I, II, and III were 57.1, 30.0, and 18.8%, respectively. Therefore, α -thal trait appears to be associated with normal splenic function and decreased frequency of severe bacterial infections in these patients.

INTRODUCTION

SCA among Kuwaiti Arabs is usually a mild disease as in patients from Eastern Saudi Arabia (1). This is not surprising since the original settlers of Kuwait migrated from the Najd Province of East Central Saudi Arabia in the 17th/18th centuries (2,3). The β^S mutation in this part of the Arabian Peninsula is usually found on chromosomes of haplotype #31 (Saudi Arabia/India) background and homozygotes have Hb F levels of about 15-30%. The latter is believed to be a major ameliorating factor in the clinical course of SS disease (4,5). However, there are a few patients who, in spite of an elevated Hb F, run an atypically severe course with frequent painful crisis and fulminant bacterial infections. The factors that characterise this subset have not been fully elucidated.

The spleen, because of its peculiar microvasculature and sluggish circulation, bears the brunt of the pathology (rigidity of the RBC membrane and consequent recurrent vaso-occlusion and infarction) in SS disease. Therefore, quite early (within the first two years) in the life of the patient, functional asplenia occurs, followed later (usually within the first decade) by fibrotic autosplenectomy (6,7). The natural history of this process has not been well documented in patients with high Hb F levels. It has, however, been reported that they maintain their splenic function till an older age compared to patients with low fetal Hb (8,9).

In the present study, splenic function was evaluated using ^{99m}Tc -labelled tin colloid and heat-denatured RBC scintigraphy. The patterns obtained were correlated with the following parameters: Age, frequency of painful crises, frequency of bacterial infection necessitating hospitalisation, haematological data (Hb, MCV, MCH, MCHC), Hb F levels, β^S haplotype, and α -globin gene patterns. Plasma ferritin was also determined to find out if iron deficiency played a role in some of the patients with microcytosis and hypochromia.

MATERIALS AND METHODS

The subjects of this study were SS patients being followed at the paediatric haematology clinics of the Mubarak Al-Kabeer and Al-Amiri hospitals in Kuwait. Informed consent was obtained from the parents of all patients. Clinical histories and patients' charts were reviewed to document the frequency of acute events necessitating hospitalisation, especially painful crisis and bacterial infections, since the diagnosis of SS disease was made. All the patients except one, who had acute splenic sequestration, were in steady state at the time of the study. None had any other chronic illness apart from SS disease.

About 5 ml of blood was obtained by venepuncture into vacutainers with EDTA as anticoagulant. Complete blood counts were obtained with an electronic cell counter (Coulter S). Fresh haemolysate was prepared from each sample and subjected to IEF (10) and cation exchange HPLC (11,12) to quantitate Hbs A, S, F, and A₂. Patients with patterns not consistent with Hb SS were excluded from the study.

The fresh blood was centrifuged and the plasma separated and kept frozen at -70°C until plasma ferritin was determined using a radioimmunoassay method (Bio-Rad Laboratories, Richmond, CA, USA). DNA was extracted from leukocytes by the method of Poncz et al (13). The β^S -globin gene cluster haplotypes were determined by hybridisation of amplified DNA, dot-blotted onto nylon membranes, with ECL-labelled synthetic oligonucleotide probes. These probes are specific for characteristic mutations in the G_{γ} and A_{γ} promoters of chromosomes with the Bnin (#19), Saudi Arabia/India (#31), and Bantu (#20) haplotypes. Details of the methodology have been previously described (13-15).

The α -globin gene patterns were determined by screening for the $-\alpha(3.7\text{ kb})$ deletion using a modified PCR method (16). All the samples were also screened for the $\alpha 2$ poly A signal mutation ($\text{AATAAA} \rightarrow \text{AATAAG}$) and the 5' IVS-I splice junction $\text{GAGGTGAGG} \rightarrow \text{GAGG}$ pentanucleotide (5 nt) deletion that are prevalent in this region, using hybridisation of amplified DNA with ECL-labelled specific synthetic oligonucleotides as previously reported (15).

Liver/spleen scintigraphs were performed with $^{99\text{m}}\text{Tc}$ -labelled tin colloid (Amersham International, plc, Amersham, Buckinghamshire, England) in all patients (17,18). Radionuclide images of the posterior, left lateral and anterior views of the splenic area were obtained in all studies. The results were graded as normal if the splenic visualisation was of the same intensity as the liver image (group I), partial if there was a decrease in the splenic image (group II) or no visualisation (group III). Patients in groups I and II were restudied at least 2 days later with heat-denatured $^{99\text{m}}\text{Tc}$ RBC (Amersham) scintigraphy (18,19).

Data are presented as mean $\pm$ SD except where otherwise stated. Student's "t" test, analysis of variance (ANOVA) or Chi-square test was used, as appropriate, to test statistical significance of differences between mean values or proportions in different groups. Analysis was with Statgraphics version 6.0 IBM-compatible PC software.

RESULTS

There were 20 SS patients (13 boys, seven girls) aged 2 to 12 years (mean 6.4 ± 2.5 years) in the study. Their individual haematological and other data are shown in Table 4a/1. Seven (35%) patients had normally visualised spleens (group I), five (25%) had partial visualisation (group II), while eight (40%) were not visualised (group III) on $^{99\text{m}}\text{Tc}$ tin colloid liver/spleen scans. All those in group II and one in group III had normal splenic visualisation on $^{99\text{m}}\text{Tc}$ heat-denatured RBC scan.

Table 4a/2 shows the mean values of different parameters in the three groups. There is a sequential increase in the MCV and MCH but a decrease in Hb and Hb F from groups I to III. Analysis of variance (ANOVA) showed that the differences between the values in the three groups were only significant for MCV ($p < 0.01$). However, when the mean values are compared between groups I and III there was significant difference for MCV ($p < 0.0001$) and MCH ($p < 0.05$).

TABLE 4a/1. Individual Haematological and Other Data

No.	Sex-Age (years)	β -Globin Haplotype	Hb g/dl	MCV fl	MCH pg	MCHC g/dl	Hb F %	α -Globin Pattern	Pain Crises	Infection
Group I										
1	F-5	31/31	8.8	80.4	19.8	29.2	24.0	- $\alpha/\alpha\alpha$	++ ^a	-
2	M-7	31/31	9.9	73.2	23.8	26.5	24.8	- α/α -5nt α	+	+
3	M-12	31/31	10.2	74.9	20.2	27.0	29.4	- $\alpha/\alpha\alpha$	++	-
4	M-3	31/31	8.1	79.9	25.1	31.5	28.0	$\alpha\alpha/\alpha\alpha$	-	-
5	F-6	31/31	8.2	65.9	18.8	28.5	15.2	- $\alpha/-\alpha$	+	-
6	M-3	31/31	10.3	74.6	25.7	31.5	27.6	- $\alpha/\alpha\alpha$	-	-
7	M-9	31/31	8.9	70.1	23.1	32.9	15.0	$\alpha\alpha/\alpha$ -5nt α	+	-
Group II										
8	M-6	31/31	9.4	92.1	27.6	31.6	28.0	$\alpha\alpha/\alpha\alpha$	+	+
9	M-7	31/31	8.2	94.0	29.7	27.5	28.0	$\alpha\alpha/\alpha\alpha$	+	-
10	M-3	31/19	9.1	77.0	26.2	32.2	19.9	$\alpha\alpha/\alpha\alpha$	+	-
11	M-3	31/31	8.9	95.3	26.2	27.0	26.2	- $\alpha/-\alpha$	+	-
12	F-7	31/31	10.6	63.0	19.2	25.4	14.7	- $\alpha/\alpha\alpha$	++	-
Group III										
13	M-8	31/31	8.1	93.7	28.4	30.6	24.8	- $\alpha/\alpha\alpha$	+	+
14	F-4	31/31	10.6	97.1	24.0	24.8	20.4	- $\alpha/\alpha\alpha$	+	-
15	F-8	31/31	8.7	97.1	31.3	32.2	13.9	$\alpha\alpha/\alpha\alpha$	+	+
16	M-10	31/31	7.3	81.5	21.0	25.8	16.1	- $\alpha/\alpha\alpha$	++	-
17	M-9	31/31	9.3	87.2	27.4	31.2	17.2	$\alpha\alpha/\alpha\alpha$	-	-
18	F-9	31/31	9.4	89.0	28.0	31.4	24.2	$\alpha\alpha/\alpha\alpha$	-	+
19 ^b	F-4	20/20	n.d.	n.d.	n.d.	n.d.	0.4	$\alpha\alpha/\alpha\alpha$	++	+
20	M-6	31/31	9.6	94.5	32.5	34.4	30.6	$\alpha\alpha/\alpha\alpha$	++	-

^a Hospital admissions: ++ + = > 10; + + = 5-10; + = 1-4.^b On chronic transfusion therapy (see text for details).

TABLE 4a/2. Mean $\pm$ SD Age and Haematological Data in the Three Groups

Groups	Age years	Hb g/dl	MCV fl	MCH pg	MCHC g/dl	Hb F %
I (n = 7)	6.4 $\pm$ 3.3	9.2 $\pm$ 0.9	74.1 $\pm$ 5.1	22.4 $\pm$ 2.7	29.6 $\pm$ 2.4	23.4 $\pm$ 6.0
II (n = 5)	6.2 $\pm$ 2.0	9.2 $\pm$ 0.9	84.3 $\pm$ 14.0	25.3 $\pm$ 3.9	29.5 $\pm$ 2.9	23.4 $\pm$ 5.9
III (n = 8)	7.0 $\pm$ 2.2	8.5 $\pm$ 1.7	90.1 $\pm$ 6.6	27.5 $\pm$ 4.0	30.1 $\pm$ 3.5	21.0 $\pm$ 5.9
All (n=20)	6.4 $\pm$ 2.5	9.0 $\pm$ 1.3	83.1 $\pm$ 10.8	25.2 $\pm$ 4.1	29.7 $\pm$ 2.8	22.5 $\pm$ 5.7

β^S Haplotyping showed that all the patients were homozygous for haplotype #31 except patient #10 (Table 4a/1) who was heterozygous for haplotypes #19 and #31, and patient #19 who was homozygous for the Bantu (#20) haplotype. The latter had a severe clinical course, with multiple acute episodes, mostly pain crisis and severe anaemia. She also had the lowest Hb F (0.4%) value in the whole group. She was, at the time of the study, on chronic transfusion therapy. Her haematological values were not included in computing the means for the study population and are not shown in Table 4a/1.

The α -globin gene pattern was successfully determined in all 20 patients. Of these, nine (45%) had a full complement ($\alpha\alpha/\alpha\alpha$), seven (35%) were α -thal-2 heterozygotes ($-\alpha^{-3.7}/\alpha\alpha$), two (10%) were α -thal-2 homozygotes ($-\alpha^{-3.7}/-\alpha^{-3.7}$), while one (5%) was a compound heterozygote for α -thal-2 and the IVS-I pentanucleotide deletion ($-\alpha^{-3.7}/\alpha^{-5nt}$), and one (5%) was a simple heterozygote for the pentanucleotide deletion ($\alpha\alpha/\alpha^{-5nt}$). Thus, the frequency of α -thal determinants in the study population was 35%. However, in group I, the frequency was 57.1%, in group II 30%, and in group III 18.8%. The difference in the distribution between groups I and III is significant ($\chi^2 = 5.5$, $p < 0.05$).

None of our patients had evidence of iron deficiency, *i.e.* plasma ferritin < 12 ng/ml. The values of plasma ferritin ranged from 21 to $\sim 1,000$ ng/ml. The highest value was in the haplotype #20 patient who was on chronic transfusion therapy.

Six (30%) patients (five males, one female; aged 3 to 12 years) had spleens palpable 3-13 cm below the left costal margin. The 3-year-old boy with the largest spleen (13 cm) had acute splenic sequestration at the time of the study, otherwise the others were in steady state. There was no significant difference between the mean age, Hb, MCV, MCH, or Hb F of this group compared to the values in those without palpable spleens. They were all homozygous for haplotype #31 except for the boy with acute splenic sequestration who was a compound heterozygote for haplotypes #31 and #19. The α -globin gene status determination showed that two were $\alpha\alpha/\alpha\alpha$, and three were α -thal-2 heterozygotes ($-\alpha/\alpha\alpha$), while one was an α -thal-2 homozygote ($-\alpha/-\alpha$). In two patients there was normal colloid uptake, while in three the spleen was partially visualised on colloid scan, but showed normal denatured RBC uptake. One patient showed no uptake on both colloid and denatured RBC scintigraphy.

One patient each in groups I and II had been hospitalised because of one episode of pneumonia, while in group III, four (50%) had had severe infections (pneumonia, osteomyelitis, lung abscess, and pyelonephritis). Frequency of infection and pain crisis are shown in Table 4a/1. One patient (#15; 8 years old) in this group has had pneumonia (three episodes), lung abscess, and pyelonephritis. Most patients in the three groups had been hospitalised on several occasions because of severe pain crisis.

DISCUSSION

Splenic dysfunction is believed to contribute significantly to the predisposition to bacterial infections in SS disease. However, its role in patients with high Hb F, who generally have a milder clinical course, has not been adequately investigated. It is interesting, therefore, that in the present study, the patients who have had recurrent severe bacterial infections are mostly in the group with poor splenic function, as shown by labelled colloid and heat-denatured RBC scans.

The conventional scintigraphy method of demonstrating functional asplenia has been the labelled-colloid uptake method. However, in a previous study from Kuwait, Owunwanne et al (17) showed that in a group of seven SS patients, aged 6 to 20 years, in whom the spleen was either not demonstrable or partially visualised on colloid uptake, it was well visualised on a heat-denatured RBC scan. This observation has also been reported in a patient (with idiopathic thrombocytopenia, post-splenectomy) whose residual spleen function was not demonstrable on colloid uptake, but well-defined on heat-denatured RBC uptake. This probably reflects the different mechanisms of uptake of colloid and denatured RBCs. Splenic uptake of radiopharmaceuticals is accomplished by the phagocytic function of reticuloendothelial cells in removing particulate matter from the circulation, while the uptake of denatured RBCs may be more reflective of the filtration function of the splenic red pulp (17,19). The present cross-sectional study shows that there is a progression from normal uptake of colloid to partial or no uptake of colloid, but normal denatured RBC uptake, and finally to the severe situation where both scanning techniques are negative. It is not known whether this is the normal sequence of events in individual SS patients; a prospective study, currently under way, will hopefully clarify this.

The splenic dysfunction in SS disease develops from a blockage of the small inter-endothelial slits in its sinuses by the rigid or sickled red cells (7). It is plausible that this process depends to some extent on the size of the cells, especially in SS disease where the red cells tend to be rigid and non-pliable. It is therefore interesting that the most significant differentiating factor among the patients with non-visualised spleens on colloid and denatured RBC uptakes in the present study is the MCV. While none of the patients had evidence of iron deficiency, the frequency of α -thal among patients with normal visualisation was 57.1% and only 18.8% in those with no visualisation.

Babiker et al (8) studied two groups of SS children from Saudi Arabia; one group of 25 from the southwestern region had low Hb F (5.6-10%), while the other group of 10 from the eastern region had high Hb F (16-25%). Eighty-four percent of the first group had no splenic colloid uptake, while in the second group, 80% had normal uptake. In another study from Eastern Saudi Arabia, Mallouh et al (9) found that among 15 SS children, 13 had either normal or partially visualised spleens on colloid uptake. In our study, only 65% of the children had normal or partially visualised spleens on colloid uptake. The factors responsible for the higher proportion of our patients with relatively poor phagocytic splenic function is not clear. It would, however, be interesting to compare the frequencies of α -thal trait in the Kuwaiti and Saudi SS populations.

Co-existent α -thal is a recognised ameliorating factor in SS patients with low Hb F (19,20). It decreases the rate of haemolysis by decreasing the MCHC, thereby resulting in higher Hb, PCV, and RBC values. Complications such as leg ulcers, renal pathology, and strokes are fewer, but the frequency of other complications, e.g. osteonecrosis and retinopathy, may be increased (22-25). Overall survival may also be enhanced (23,26). The present study is probably the first to recognise an association of α -thal trait with preserved splenic function in these patients.

Acknowledgements. The authors express their gratitude to Professor Dr. Titus H.J. Huisman of the Medical College of Georgia, Augusta, GA, USA for assistance with HPLC analysis, the α -globin gene pattern determinations, and for reviewing the manuscript. The technical assistance of Dr. K. Nawaz and Ms. Tagreed Yacoub and the secretarial assistance of Mrs. Marianne F.H. Carver are appreciated. This study was supported, in part, by Kuwait University Research Grant No. MK031.

REFERENCES

1. Ali, S.: Milder variant of sickle-cell disease in Arabs associated with unusually high level of foetal haemoglobin. *Br. J. Haematol.*, 19:613, 1970.
2. Abu-Hakima, A.M.: *The Modern History of Kuwait*, Luzac & Co. Ltd., London, 1983.
3. Kutlar, A., Hattori, Y., Bakioglu, I., Kutlar, F., Kamel, K., and Huisman, T.H.J.: Hematological observations in Arabian SS patients with a homozygosity or heterozygosity for a β^S chromosome with haplotype #31. *Hemoglobin*, 9:545, 1985.
4. Miller, B.A., Olivieri, N., Salameh, M., Ahmed, M., Antognetti, G., Huisman, T.H.J., Nathan, D.G., and Orkin, S.H.: Molecular analysis of the high-Hemoglobin F phenotype in Saudi Arabian sickle cell anemia. *N. Engl. J. Med.*, 316: 244, 1987.
5. Powars, D.R., Chan, L., and Schroeder, W.A.: β^S -Gene cluster haplotypes in sickle cell anemia: Clinical implications. *Am. J. Pediatr. Hematol./Oncol.* 12: 367, 1990.
6. Pearson, H.A., McIntosh, S., Richey, A.K., Lobel, J.S., Rooks, Y., and Johnson, D.: Developmental aspects of splenic function in sickle cell disease. *Blood*, 53: 358, 1979.
7. Serjeant, G.R.: The spleen. In: *Sickle Cell Disease*, 2nd edition, page 135, Oxford University Press, Oxford, 1992.
8. Babiker, M.A., El-Hazmi, M.A.F., Al-Jabori, A.M., Obeid, H., and Bahakim, H.M.: Splenic function in children with sickle cell disease: Two different patterns in Saudi Arabia. *Scand. J. Haematol.*, 35:191, 1985.
9. Mallouh, A., Burke, G.M., Salameh, M., and Ahmad, M.S.: Splenic function in Saudi children with sickle cell disease. *Ann. Trop. Paediatr.*, 4:87, 1984.
10. Righetti, P.G., Gianazza, E., Bianchi-Bosisio, A., and Cossu, G.: Conventional isoelectric focusing and immobilized pH gradients for hemoglobin separation and identification. In: *The Hemoglobinopathies*, edited by T.H.J. Huisman, Methods in Hematology, Vol. 15, page 47, Churchill Livingstone, Edinburgh, 1986.

11. Bissé, E. and Wieland, H.: High-performance liquid chromatographic separation of human haemoglobins. Simultaneous quantitation of foetal and glycated haemoglobins. *J. Chromatogr.*, 434:95, 1988.
12. Kutlar, A., Kutlar, F., Gu, L-G., Mayson, S.M., and Huisman, T.H.J.: Fetal hemoglobin in normal adults and β -thalassemia heterozygotes. *Hum. Genet.*, 85:106, 1990.
13. Poncz, M., Solowiejczyk, D., Harpel, B., Mory, Y., Schwartz, E., and Surrey, S.: Construction of human gene libraries from small amounts of peripheral blood: Analysis of β -like globin genes. *Hemoglobin*, 6:27, 1982.
14. Dimovski, A.J., Öner, C., Agarwal, S., Gu, Y-C., Kutlar, F., Lanclos, K.D., and Huisman, T.H.J.: Certain mutations observed in the 5' sequences of the G_γ - and A_γ -globin genes of β^S chromosomes are specific for chromosomes with major haplotypes. *Acta Haematol.*, 85:79, 1991.
15. Adekile, A.D., Gu, L-H., Baysal, E., Haider, M.Z., Al-Fuzae, L., Aboobacker, K.C., Al-Rashied, A., and Huisman, T.H.J.: Molecular characterization of α -thalassemia determinants, β -thalassemia alleles, and β^S haplotypes among Kuwaiti Arabs. *Acta Haematol.*, 92:176, 1994.
16. Baysal, E. and Huisman, T.H.J.: Detection of common deletion α -thalassemia-2 determinants by PCR. *Am. J. Hematol.*, 46:208, 1994.
17. Owunwanne, A., Halkar, R., Al-Rashied, A., Aboobacker, K.C., and Abdel-Dayem, H.: Radionuclide imaging of the spleen with heat denatured technetium-99m RBC when the splenic reticuloendothelial system seems impaired. *J. Nuclear Med.*, 29:320, 1988.
18. Massey, M.D. and Stevens, J.S.: Residual spleen found on denatured red blood cell scan following negative colloid scans. *J. Nuclear Med.*, 32:2286, 1991.
19. Embury, S.H., Clark, M.R., Monroy, G., and Mohandas, N.: Concurrent sickle cell anemia and α -thalassemia: Effect on pathologic properties of sickle erythrocytes. *J. Clin. Invest.*, 73:116, 1984.
20. Bowdler, A.J.: Splenomegaly and hypersplenism. In: *Clinics in Haematology*, edited by S.M. Lewis, Vol. 12, page 478, W.B. Saunders Company, London, 1983.
21. Higgs, D.R., Adlridge, B.E., Lamb, J., Clegg, J.B., Weatherall, D.J., Hayes, R.J., Grandison, Y., Lowrie, Y., Mason, K.P., Serjeant, B.E., and Serjeant, G.R.: The interaction of alpha-thalassemia and homozygous sickle-cell disease. *N. Engl. J. Med.*, 306:1441, 1982.
22. Nagel, R.L.: Severity pathobiology, epistatic effect and genetic markers in sickle cell anemia. *Semin. Hematol.*, 28:108, 1991.
23. Mears, J.G., Lachman, H.M., Labie, D., and Nagel, R.L.: α -Thalassemia is related to prolonged survival in sickle cell anemia. *Blood*, 62:286, 1983.
24. Steinberg, M.H., Rosenstock, W., Coleman, M.B., Adams, J.G., Platika, O., Cedeno, M., Rieder, R.F., Wilson, J.T., Milner, P., West, S., and the Cooperative Study of Sickle Cell Disease: Effects of thalassemia and microcytosis on the hematologic and vasoocclusive severity of sickle cell anemia. *Blood*, 63: 1353, 1984.
25. Adams, R.J., Kutlar, A., McKie, V., Carl, E., Nichols, F.T., Liu, J-C., McKie, K., and Clary, A.: Alpha thalassemia and stroke risk in sickle cell anemia. *Am. J. Hematol.*, 45:279, 1994.

26. Adekile, A.D., Liu, J-C., Sulzer, A.J., and Huisman, T.H.J.: Frequency of the α -thalassemia-2 gene among Nigerian SS patients and its influence on malaria antibody titers. Hemoglobin, 17:73, 1993.

CHAPTER 4b

**MORBIDITY, β^S HAPLOTYPE, AND α -GLOBIN GENE PATTERNS
AMONG SS PATIENTS IN KUWAIT**

A.D. Adekile and M.Z. Haider

Department of Paediatrics, Faculty of Medicine
Kuwait University, Safat, Kuwait 13110

(Adapted from: *Acta Haematol.*, in press)

ABSTRACT

Admission records of children with SS disease, in the two main teaching hospitals in Kuwait, were reviewed for a 1-year period. The haplotypes of 92 β^S chromosomes (from 39 SS, 11 AS, two S- β -thal, and one SD individuals) were determined using an ASO hybridisation technique, and the α -globin gene status of 27 SS and 33 AS individuals, *i.e.* 120 chromosomes, were determined with a combination of PCR and ASO techniques. Vaso-occlusive crisis was the most common (60%) cause of hospitalisation, followed by infections (20%). Hospital admissions were most common during the hottest month of the year (July). Few complications of the disease were seen among patients on follow-up; however, splenomegaly was present in 24%, hepatomegaly in 15.2%, gallstones in 15.2%, and aseptic necrosis of the femoral head in 6.1%. Haplotype #31 (Saudi Arabia/India) is the most frequent in this community, being present in 80.4% of the chromosomes tested; the Benin haplotype #19 was found in 12%, and the Bantu haplotype #20 in 6.5%. Hb F in the haplotype #31 homozygotes and compound heterozygotes ranged from 11.4 to 35.1% (mean $22.5 \pm 5.2\%$). The frequency of α -thal determinants in the study was 40%, the commonest being the - α (3.7 kb) deletion in 27.5%, the $\alpha 2$ poly A signal (AATAAA→AATAAG) mutation (10.2%), and the IVS-I 5' end GAGGTGAGG→GAGG pentanucleotide (5 nt) deletion (3.3%). SS patients with co-existent α -thal trait did not have severe recurrent infections and none had gallstones. It appears that the high frequencies of the Saudi Arabia/India β^S haplotype and α -thal trait contribute to the mild nature of SS disease among Kuwaiti Arabs comparable to that in Eastern Saudi Arabia.

INTRODUCTION

Although Kuwait was founded by members of the Utub tribe who migrated from Najd in East Central Saudi Arabia in the 17th/18th centuries, the early settlers included people of Iraqi and Iranian ancestries (1,2). Also, a proportion (about 8%) of the indigenous population is made up of semi-nomadic Arab Bedouins whose antecedents are not always known, but have been traced to various parts of the Middle East (Western Saudi Arabia, Syria, Iraq, Jordan, etc.) (3,4).

Sickle cell anaemia is usually mild among Kuwaiti SS patients as has been reported for patients from Eastern Saudi Arabia (5). However, a subset of patients presents with a severe clinical course. The reasons for this are not known, but two important determinants of severity in SS disease are the haplotype of the β^S -globin gene cluster and co-existence of α -thal trait (6-9). There have been no previous studies to document these molecular characteristics among Kuwaiti patients, especially with reference to their clinical presentation. The present study was designed to document the morbidity pattern of sickle cell anaemia among Kuwaiti patients and relate this to the prevalent haplotype(s) and α -globin gene patterns.

MATERIALS AND METHODS

Admission records in the paediatric wards of the two major teaching hospitals in Kuwait (Mubarak Al-Kabeer and Al-Amiri) for a 1-year period (January to December 1993) were retrospectively reviewed. The details of all hospitalised SS patients (diagnosis, mode of treatment, length of stay in the hospital, etc.) were documented.

Secondly, all SS patients referred for follow-up in the paediatric haematology clinics of the two hospitals from October 1993 to June 1995 were studied. Most were in steady state at the time of the study. Informed consent was obtained from the parents of all patients. At recruitment, clinical histories, and patients' charts were reviewed to document the frequency of acute events necessitating hospitalisation, especially painful crises, bacterial infections, and any complications of the disease.

Apart from other routine procedures, about 5 ml of blood was obtained by venepuncture into vacutainers with EDTA as anticoagulant. Complete blood counts were obtained with an electronic cell counter (Coulter S). Fresh haemolysate was prepared from each sample and subjected to IEF (13) and cation exchange HPLC (14,15) to quantitate Hbs A, S, F, and A₂.

DNA was extracted from leucocytes by the method of Poncz et al (16). The β^S -globin gene cluster haplotypes were determined by hybridisation of amplified DNA, dot-blotted onto nylon membranes with enhanced ECL-labelled synthetic oligonucleotide probes. These probes are specific for certain mutations in the G γ and A γ promoters of the Benin (#20), Saudi Arabia/India (#31), and Bantu (#20) haplotypes. Details of the methodology have been previously described (17,18).

The α -globin gene patterns were determined by screening for the 3.7 kb deletion using a modified Baysal and Huisman PCR method (19). All the samples were also screened for the $\alpha 2$ poly A signal mutation (PA-1 or AATAAA $\rightarrow$ AATAAG) and the IVS-I GAGGTGAGG $\rightarrow$ GAGG pentanucleotide (5 nt) deletion, that are prevalent in this region, using hybridisation techniques with ECL-labelled specific synthetic oligonucleotides as previously reported (18).

All patients had abdominal ultrasonography to detect gallstones. Spleen function was also investigated using ^{99m}Tc -labelled tin colloid scintigraphy (10,11); these results are being reported separately (12).

RESULTS

During the period under review, 25 SS patients (16 males, nine females) were admitted on 50 occasions for various acute illnesses. Their ages ranged from 1.5 to 12 years, with a mean of 7.1 ± 2.7 years. There were 58 different diagnoses; 35 (60.3%) presented with vaso-occlusive crisis, 11 (19%) with different types of infections (four pneumonia, two each with cellulitis and osteomyelitis, one each with lung abscess, arthritis, and hepatitis), nine (15.8%) with haemolytic crisis, and three (5.2%) with hypersplenism. The admissions were spread evenly throughout the year with two to four admissions each month, except in May and July with seven and 10 admissions, respectively. Four patients were admitted on four or five occasions each. One patient was admitted for pneumonia on one occasion, and two months later presented with acute osteomyelitis. She also had a past history of septicaemia and disseminated intravascular coagulopathy. Another patient, who was admitted for pneumonia, later presented with a lung abscess. She also has had recurrent pyelonephritis. No SS patient died during the study period.

Of the 33 SS patients being followed in the clinics, eight (24%), aged 3 to 12 years (mean of 7.5 ± 2.7 years), had palpable spleens of 3-13 cm below the left costal margin. The 3-year-old with the largest spleen had acute splenic sequestration at the time of the study, whereas the others were in steady state and their spleens had remained palpable for at least 6 months. The mean Hb and RBC values were lower in the latter in comparison to those without splenomegaly, although the differences were not statistically significant. The liver was palpable in five (15.2%) patients, aged 3 to 8 years (four males, one female). Abdominal ultrasonography revealed gallstones in five (15.2%); three females and two males with ages ranging from 7 to 13 years (mean of 10.8 ± 2.4 years). Necrosis of the femoral head was present in two (6.1%) patients, one male and one female, aged 10 and 13 years, respectively. Only one (aged 2 years) presented with mild hand and foot swelling. No patient had marked skull bossing or gnathopathy.

β^S Haplotyping was successfully carried out in 39 SS patients; 11 AS, two S- β -thal, and one SD individual, *i.e.* 92 β^S chromosomes. Seventy-four (80.4%) carried haplotype #31 (Saudi Arabian/Indian) mutations, 11 (12%) had haplotype #19 (Benin). Six (6.5%) chromosomes, all from the same Bedouin family, had haplotype #20 (Bantu), while one (1.1%) was a hybrid chromosome. The latter, the details of which

have been previously reported, had haplotype #19 characteristics in the G_γ promoter region, but haplotype #31 characteristics in the β -globin gene LCR. Of the 39 SS patients, 30 (76.9%) were homozygous for haplotype #31, six (15.4%) were compound heterozygotes for haplotypes #31 and #19, while one was homozygous for haplotype #20, and one was homozygous for haplotype #19. The last patient was a compound heterozygote for haplotype #19 and the hybrid chromosome described earlier.

The α -globin gene pattern was determined in 27 SS patients and 33 AS individuals, *i.e.* a total of 120 chromosomes. The frequency of α -thal determinants in the whole group was 40%; among the SS patients it was 31.5%, and in the AS individuals 47%. The difference in this distribution is not significant ($\chi^2 = 2.2$, $p = 0.13$). The $-\alpha(3.7 \text{ kb})$ deletion was the most common determinant (27.5%), followed by the $\alpha 2$ PA-1 mutation (10.2%), and the IVS-I 5 nt deletion (3.3%).

Table 4b/1 shows some haematological data on the SS patients in the study, grouped according to various parameters. Except for the two patients who were homozygous for haplotypes #19 and #20, respectively, all had Hb F values ranging from 11.4 to 35.1% (mean $22.5 \pm 5.2\%$). There was no sex difference in the mean haematological values. There was no significant difference in the frequency of α -thal trait in patients with or without hepatic or splenic enlargement. However, none of the five patients with gallstones and the two patients with recurrent severe bacterial infections in the present study, had α -thal trait. Of the two patients with aseptic necrosis of the head of the femur, one had four α -globin genes ($\alpha\alpha/\alpha\alpha$) and the other was an α -thal-2 homozygote ($-\alpha/-\alpha$).

DISCUSSION

Over 90% of the SS patients in the present study were either homozygous or heterozygous for the Saudi Arabia/India haplotype #31. A previously reported 5-year-old patient was heterozygous for haplotype #19 (Benin) and a hybrid chromosome with features of both haplotypes #19 and #31 at different loci within the β -globin gene cluster (18). She had a Hb F level of 30.2% and G_γ of 64.4%, and has never been hospitalised for any acute illness. The only homozygous haplotype #19 patient in the study has a Jordanian father and a Kuwaiti mother. There was one homozygous haplotype #20 (Bantu) Bedouin patient; the ancestry of her parents could not be determined; both parents and two of four siblings are heterozygous for the chromosome. To our knowledge, this is the first Arab family in whom this haplotype has been reported. The child has had a severe clinical course and is currently on chronic transfusion therapy.

The segregation of haplotype #31 in this country is similar to that of Eastern Saudi Arabia (20,21) from where the ancestors of many present-day Kuwaitis migrated. The mild clinical course of our patients is therefore not surprising. However, there had been no previous reports of the frequency of α -thal in Kuwait. It is, therefore, interesting that in our population of AS and SS patients, the frequency was 40%. This is similar to the figure of 37.5% reported by Padmos et al (22) for patients from

TABLE 4b/1. Haematological Data on SS Patients Grouped According to Sex, Haplotype, and α -Thal Status

Group	Age years	Hb g/dl	RBC $10^{12}/l$	MCV fl	MCH pg	Hb F %	G _γ %
All (n = 39)	8.0 ± 6.1	9.4 ± 1.3	3.75 ± 0.8	84.6 ± 13.5	25.2 ± 4.0	22.5 ± 5.2	69.3 ± 6.0
Males (n = 21)	7.0 ± 4.4	9.3 ± 1.2	3.54 ± 0.7	83.2 ± 15.6	24.4 ± 4.6	23.5 ± 5.5	70.0 ± 5.8
Females (n = 18)	6.5 ± 3.7	9.6 ± 1.5	4.00 ± 0.9	86.1 ± 11.3	24.4 ± 4.6	21.4 ± 4.8	68.6 ± 6.3
31/31 ^a (n = 30)	6.9 ± 3.4	9.5 ± 1.2	3.73 ± 0.8	84.9 ± 13.9	25.5 ± 3.8	22.6 ± 3.8	69.6 ± 5.0
31/19 ^a (n = 6)	7.5 ± 4.5	9.7 ± 1.0	3.38 ± 0.8	83.6 ± 13.8	26.7 ± 4.8	19.5 ± 6.3	73.3 ± 7.6
19/19 ^a (n = 1)	4.0	8.9	3.15	96.2	28.3	4.3	57.3
19/hybrid ^a (n = 1)	5.0	10.9	4.85	80.2	22.5	30.2	64.4
$\alpha\alpha/\alpha\alpha$ (n = 15)	6.9 ± 3.2	8.9 ± 1.4	3.2 ± 0.5	90.9 ± 8.9	27.5 ± 3.6	21.7 ± 4.7	69.6 ± 3.0
α -Thal ^b (n = 12)	7.0 ± 2.9	9.4 ± 1.2	3.8 ± 0.6	78.4 ± 17.8	23.1 ± 3.5	21.5 ± 4.7	66.0 ± 3.9

^a Haplotype number; values for the homozygous haplotype #20 patient are not shown because she was on chronic transfusion therapy.

^b Includes all homozygotes or heterozygotes for different α -thal determinants.

Eastern Saudi Arabia. However, they found only the $-\alpha(3.7 \text{ kb})$ deletion, but in the present study, 13.5% of the individuals had nondeletional α -thal trait.

Vaso-occlusive crisis was also reported as a common clinical feature among SS patients from Eastern Saudi Arabia (22,23), with 36% having more than three severe episodes a year. Splenomegaly was reported in 53% of cases. Dactylitis, haemolytic crisis, acute chest syndrome, and severe infections were uncommon. On the other hand, in a study of Nigerian SS children (24) in whom the predominant haplotype is #19 (Benin) (25) and frequency of α -thal is about 25% (26), the most common cause of hospitalisation is infection (pneumonia, malaria, meningitis, osteomyelitis) which was found in 37.5% of cases. Following this were haemolytic crisis (25.5%) and vaso-occlusive crisis (19.6%). The frequencies of some common associated physical features among Nigerian patients were: Splenomegaly (26%), hepatomegaly (52%), skull bossing (22%), gnathopathy (10%), digital clubbing (9%), and hand/foot swelling (8%). One environmental factor that influences the clinical course of SS disease in Nigeria and in other tropical African countries is endemic malaria which contributes to the frequency of haemolysis and splenomegaly (27,28).

Gallstones were found in five (15.2%) patients in the present study. These are usually pigment stones because of the chronic haemolysis in SS disease. It is interesting that none of the five patients has a co-existent α -thal trait. Although this number is small, it will be interesting to see if larger studies bear out the suggested protective role of α -thal in the pathogenesis of these stones. In American patients, the frequency of stones is about 30% by the age of 10 years (29). However, they are rare among Nigerian patients, in whom most studies report frequencies of less than 5% at a similar age (30,31). This raises the possibility that other factors, *e.g.* diet, might be involved in the pathogenesis of stones in SS disease. This area deserves further studies.

This and other studies (12,20,21) suggest a significant ameliorating role for α -thal in SS patients from this region. Therefore, screening for α -thal determinants should be part of the routine work-up of these patients. This will facilitate effective counselling and early identification of patients who are likely to have a severe course. While the two patients in this study, who had severe recurrent infections and a generally severe clinical course, did not have a co-existent α -thal, other (as yet unidentified) factors are probably present that adversely influence their predisposition to infections.

Acknowledgements. The authors thank Professor Allie Moosa, Chairman, Department of Paediatrics, Kuwait University, for his encouragement and support. The advice of Professor Titus H.J. Huisman, Department of Biochemistry and Molecular Biology, Medical College of Georgia, Augusta, GA, USA is highly appreciated. We are indebted to Mrs. Marianne F.H. Carver for secretarial assistance. This work was supported by Kuwait University research grant MK031.

REFERENCES

1. Abu-Hakima, A.M.: *The Modern History of Kuwait*, Luzac & Co. Ltd., London, page 1, 1983.
2. Kuwait University. *Welcome to Kuwait and Kuwait University*, Handbook, Kuwait University Press, 1992.
3. Central Statistics Office, Annual Statistical Report, Ministry of Planning, Kuwait Statistical Abstract Series, 1993.
4. Ingham, B., editor: The history of the Al Dhafir. In: *Bedouin of Northern Arabia*, page 8, KPI, London, 1986.
5. Ali, S.: Milder variant of sickle-cell disease in Arabs in Kuwait associated with unusually high level of foetal haemoglobin. *Br. J. Haematol.*, 19:613, 1970.
6. Powars, D.R., Chan, L., and Schroeder, W.A.: β^S -Gene cluster haplotypes in sickle cell anemia: Clinical implications. *Am. J. Pediatr. Hematol./Oncol.*, 12: 367, 1990.
7. Nagel, R.L.: Severity pathobiology, epistatic effect and genetic markers in sickle cell anemia. *Semin. Hematol.*, 28:108, 1991.
8. Embury, S.H., Clark, M.R., Monroy, G., and Mohandas, N.: Concurrent sickle cell anemia and α -thalassemia: Effect on pathologic properties of sickle erythrocytes. *J. Clin. Invest.*, 73:116, 1984.
9. Higgs, D.R., Aldridge, B.E., Lamb, J., Clegg, J.B., Weatherall, D.J., Hayes, R.J., Grandison, Y., Lowrie, Y., Mason, K.P., Serjeant, B.E., and Serjeant, G.R.: The interaction of α -thalassemia and homozygous sickle cell disease. *N. Engl. J. Med.*, 306:1441, 1982.
10. Pearson, H.A., McIntosh, S., Richey, A.K., Lobel, J.S., Rooks, Y., and Johnson, D.: Developmental aspects of splenic function in sickle cell disease. *Blood*, 53: 358, 1979.
11. Owunwanne, A., Halkar, R., Al-Rashied, A., Aboobacker, K.C., and Abdel-Dayem, H.: Radionuclide imaging of the spleen with heat denatured technetium-99m RBC when the splenic reticuloendothelial system seems impaired. *J. Nuclear Med.*, 29:320, 1988.
12. Adekile, A.D., Tuli, M., Haider, M.Z., Al-Zaabi, K., Al-Mohannadi, S., and Owunwanne, O.: Influence of α -thalassemia trait on spleen function in sickle cell patients with high Hb F. *Am. J. Hematol.*, in press.
13. Righetti, P.G., Gianazza, E., Bianchi-Bosisio, A., and Cossu, G.: Conventional isoelectric focusing and immobilized pH gradients for hemoglobin separation and identification. In: *The Hemoglobinopathies*, edited by T.H.J. Huisman, *Methods in Hematology*, Vol. 15, page 47, Churchill Livingstone, Edinburgh, 1986.
14. Bissé, E. and Wieland, H.: High-performance liquid chromatographic separation of human haemoglobins. Simultaneous quantitation of foetal and glycated haemoglobins. *J. Chromatogr.*, 434:95, 1988.
15. Kutlar, A., Kutlar, F., Gu, L-G., Mayson, S.M., and Huisman, T.H.J.: Fetal hemoglobin in normal adults and β -thalassemia heterozygotes. *Hum. Genet.*, 85:106, 1990.
16. Poncz, M., Solowiejczyk, D., Harpel, B., Mory, Y., Schwartz, E., and Surrey, S.: Construction of human gene libraries from small amounts of peripheral blood: Analysis of β -like globin genes. *Hemoglobin*, 6:27, 1982.

17. Dimovski, A.J., Öner, C., Agarwal, S., Gu, Y-C., Kutlar, F., Lanclos, K.D., and Huisman, T.H.J.: Certain mutations observed in the 5' sequences of the γ - and δ -globin genes of β^S chromosomes are specific for chromosomes with major haplotypes. *Acta Haematol.*, 85:79, 1991.
18. Adekile, A.D., Gu, L-H., Baysal, E., Haider, M.Z., Al-Fuzae, L., Aboobacker, K.C., Al-Rashied, A., and Huisman, T.H.J.: Molecular characterization of α -thalassaemia determinants, β -thalassaemia alleles, and β^S haplotypes among Kuwaiti Arabs. *Acta Haematol.*, 92:176, 1994.
19. Baysal, E. and Huisman, T.H.J.: Detection of common deletional α -thalassaemia-2 determinants by PCR. *Am. J. Hematol.*, 46:208, 1994.
20. Kutlar, A., Hattori, Y., Bakioglu, I., Kutlar, F., Kamel, K., and Huisman, T.H.J.: Hematological observations on Arabian SS patients with a homozygosity or heterozygosity for a β^S chromosome with haplotype #31. *Hemoglobin*, 9:545, 1985.
21. Miller, B.A., Olivieri, N., Salameh, M., Ahmed, M., Antognetti, G., Huisman, T.H.J., Nathan, D.G., and Orkin, S.H.: Molecular analysis of the high-Hemoglobin F phenotype in Saudi Arabian sickle cell anemia. *N. Engl. J. Med.*, 316: 244, 1987.
22. Padmos, M.A., Roberts, G.T., Kulozik, A., Bail, S., Morris, J.S., Serjeant, B.E., and Serjeant, G.R.: Two different forms of homozygous sickle cell disease occur in Saudi Arabia. *Br. J. Haematol.*, 79:93, 1991.
23. El-Hazmi, M.A.F.: Clinical manifestations and laboratory findings of sickle cell anaemia in association with α -thalassaemia in Saudi Arabia. *Acta Haematol.*, 74:155, 1985.
24. Adekile, A.D.: Morbidity pattern in SS patients in Ile-Ife, Nigeria (unpublished data).
25. Adekile, A.D., Kitundu, M.N., Gu, L-H., Adeodu, O.O., Lanclos, K.D., and Huisman, T.H.J.: Haplotypes in SS patients from Nigeria; characterization of one atypical β^S haplotype #19 (Benin) associated with elevated Hb F and high γ levels. *Ann. Hematol.*, 65:41, 1992.
26. Adekile, A.D., Liu, J-C., Sulzer, A.J., and Huisman, T.H.J.: Frequency of the α -thalassaemia-2 gene among SS patients from Nigeria and its influence on malaria antibody titers. *Hemoglobin*, 17:73, 1993.
27. Adekile, A.D., McKie, K.M., Adeodu, O.O., Sulzer, A.J., McKie, V.C., Kutlar, F., Ramachandran, M., Kaine, W., Akenzua, G.I., Akolo, A.A., Asindi, A.A., Obinyan, E.A., Ogala, W.N., Ibrahim, M., and Huisman, T.H.J.: The spleen in sickle cell anemia: Comparative studies of Nigerian and U.S. patients. *Am. J. Hematol.*, 42:316, 1993.
28. Adekile, A.D., Adeodu, O.O., Jefe, A.O., and Odesanmi, W.O.: Persistent splenomegaly in sickle cell disease: Relationship to malaria. *Ann. Trop. Paediatr.*, 8:103, 1988.
29. Serjeant, G.R.: Cholelithiasis. In: *Sickle Cell Disease*, 2nd edition, page 129, Oxford University Press, Oxford, 1992.
30. Adekile, A.D. and Mekanjuola, D.I.: Realtime gallbladder ultrasonography in children with sickle cell anaemia. *Nig. J. Paediatr.*, 10:35, 1983.
31. Adekile, A.D.: Experience with cholelithiasis in Nigerian sickle cell patients. *Am. J. Pediatr. Hematol./Oncol.*, 7:261, 1985.

CHAPTER 4c

MOLECULAR CHARACTERIZATION OF α -THALASSAEMIA DETERMINANTS,
 β -THALASSAEMIA ALLELES, AND β^S HAPLOTYPES
 AMONG KUWAITI ARABS

A.D. Adekile¹, L.H. Gu², E. Baysal¹, M.Z. Haider¹, L. Al-Fuzae³,
 K.C. Aboobacker³, A. Al-Rashied³, and T.H.J. Huisman²

¹ Department of Paediatrics, Faculty of Medicine, Kuwait University, Kuwait

² Department of Biochemistry and Molecular Biology
 Medical College of Georgia, Augusta, GA 30912-2100, USA

³ Department of Paediatrics, Al-Sabah Hospital, Kuwait

(Adapted from: *Acta Haematol.*, 92:176-181,1994)

ABSTRACT

Using amplification, ASO hybridization, and DNA sequencing we have documented the molecular basis of 64 α - and 123 β -thal chromosomes, and the haplotypes of 18 β^S chromosomes from patients followed in three hospitals in Kuwait. Of the 30 chromosomes from 15 patients with Hb H disease, 26 (86.7%) carried the poly A signal mutation (AATAAA→AATAAG) in the $\alpha 2$ -globin gene, three (10%) had the - α (3.7 kb) deletion, and one (3.3%) had the pentanucleotide deletion in the 5' IVS-I splice junction ($\alpha^{-5nt}\alpha$). As many as 12 different β -thal mutations were identified; six Mediterranean alleles [IVS-I-1 (G→A), IVS-I-6 (T→C), CD 39 (C→T), IVS-I-110 (G→A), CD 8 (-AA), and IVS-I-6 (G→A)] were present in 79 (64.2%) of the chromosomes tested. Four East Indian alleles [IVS-I-6 (G→C), IVS-I 3' end 25 nt deletion, CDs 8/9 (+G), and 619 bp deletion] were found in 31 (25%), and the two Kurdish/Iranian alleles [CD 44 (-C) and CDs 36/37 (-T)] were found in 13 (10.6%) chromosomes. Fourteen β^S chromosomes carried haplotype #31 (Saudi Arabia/India); three had haplotype #19 (Benin), and one was a hybrid with haplotype #31-specific characteristics in the LCR-HS-2, and haplotype #19-specific mutations in the 5' flanking region of the G_γ promoter. The patient homozygous for haplotype #19 was a Jordanian, while the others were Kuwaiti Arabs. The latter appear to be fairly homogeneous in terms of the prevalent α -thal determinants and β^S haplotypes, but there is considerable heterogeneity of their β -thal alleles. This has implications for genetic counselling and prenatal diagnosis programs.

INTRODUCTION

The State of Kuwait lies at the northeast extremity of the Arabian Peninsula. To the north and west it shares a border with the Republic of Iraq, on the south and southwest with the Kingdom of Saudi Arabia, and on the east is the Arabian Gulf. In 1993, the total population was estimated at 1,698,077, of whom 43% were Kuwaitis, 49% expatriates, and 8% of undetermined statehood (Bedouins). Kuwait was founded by the Utub tribe, which migrated from the Al-Aflaj region in Najd, Central Saudi Arabia in the early part of the 18th century, although the early settlers included people of Iranian and Iraqi ancestry (1).

α -Thal, β -thal, and SS are the major hemoglobinopathies in the Arabian Gulf. The following frequencies have been reported for some of the other countries in the region: Oman 0.389, 0.024, 0.038; United Arab Emirates 0.165, 0.017, 0.019, and Yemen 0.065, 0.024, 0.0095 for α -thal, β -thal, and β^S traits, respectively (2). In Saudi Arabia, the figures vary considerably depending upon the province studied (3,4). These haemoglobinopathies have not been systematically studied in Kuwait. The present study, therefore, was designed to determine the prevalent α - and β -thal alleles and the β^S haplotypes in this population, relate these to the patients' ancestral origins, and compare the mutations to those found in neighbouring countries.

MATERIALS AND METHODS

The patients were followed in the paediatric haematology clinics of the Al-Sabah, Mubarak Al-Jabeer, and Al-Amiri Hospitals, and resided within the Kuwait City metropolis. They were consecutive patients seen in a 3-month period (October to December 1993). In many instances, family members of the patients were also studied. Attempts were made to ascertain the ancestral origin(s) of each family. Informed consent was obtained.

Blood was collected in vacutainers with EDTA as anticoagulant and transported by fast courier service to Augusta, GA (USA). Complete blood counts and red cell indices were obtained with an electronic cell counter (Sysmex, Kobe, Japan). All samples were analysed by IEF (5), and by cation exchange HPLC to quantitate Hbs A, A₂, F, and S (6,7). Hb F was isolated from the samples by DEAE-cellulose chromatography (8) and its γ chain composition determined by reversed phase HPLC (9,10). DNA was isolated from peripheral leukocytes as described by Poncz et al (11). PCR amplification of β - and α -globin gene regions was carried out as previously described (12-14). Identification of the different β - and α -thal mutations was by dot-blot analysis of amplified DNA, and hybridisation to ^{32}P - α -labelled specific synthetic oligonucleotides. Some of the β -thal mutations were detected with the dideoxy chain termination sequencing method of Sanger et al (15). β^S Haplotyping was carried out by identifying haplotype-specific mutations in the G γ and A γ promoter regions (16-18). Deletional α -thal-2 determinants were identified using a PCR-based method (19). All Hb H cases were also screened for the following mutations using ASO hybridisation of the amplified $\alpha 2$ -globin gene: Poly A ($\alpha^{\text{PA-1}}$) (AATAAA $\rightarrow$ AATAAG), termination CD (Hb

Icaria; TAA→AAA), initiation CD (ATG→ACG), and the 5' IVS-I pentanucleotide deletion ($\alpha^{-5nt}\alpha$) (GAGGTGAGG→GAGG).

RESULTS

α -Thal. Fifteen patients from seven unrelated families with Hb H disease were studied along with 17 of their immediate relatives (parents and siblings). They were Kuwaiti Arabs whose ancestral origin could be established in five families; two had migrated from Saudi Arabia, while three originally came from Iran. The probands (11 males, four females) ranged in age from 3 months to 15 years. All had hypochromic (MCH 14.3-18.4 pg), microcytic (MCV 52.6-78.4 fl) anemia (Hb 6.7-11.3 g/dl) with the typical gross disruption of red cell morphology. Hb H was observed by electrophoresis and inclusion bodies were readily detected. None were transfusion-dependent but most had been transfused sporadically, and all had varying degrees of hepatosplenomegaly.

PCR and ASO hybridisation studies showed that 11 of the Hb H patients were homozygous for the poly A mutation ($\alpha^{PA-1}\alpha/\alpha^{PA-1}\alpha$). Three were compound heterozygotes for the poly A mutation and the $-\alpha(3.7\text{ kb})$ deletion ($\alpha^{PA-1}\alpha/\alpha^{3.7}$), while one had the poly A mutation on one chromosome and the pentanucleotide deletion on the other ($\alpha^{PA-1}\alpha/\alpha^{-5nt}\alpha$). Of the eight parents available for the study, five were heterozygous and 2 were homozygous for the poly A mutation, and one was heterozygous for the pentanucleotide deletion. Pedigrees of two families are shown in Fig. 4c/1. Table 4c/1 gives the mean haematological values for individuals with various α -thal genotypes; the values for eight normals ($\alpha\alpha/\alpha\alpha$) are included for comparison.

TABLE 4c/1. Haematological Values in Individuals With Various α -Globin Genotypes (average values only)^a

Genotype	n	Hb g/dl	RBC 10 ¹² /l	MCV fl	MCH pg	Hb A ₂ %
$\alpha\alpha/\alpha\alpha$	8	14.1	5.13	93.5	27.4	2.6
$\alpha\alpha/\alpha^{PA-1}\alpha$	7	13.2	5.40	85.1	24.7	2.5
$\alpha\alpha/\alpha^{3.7}$	4	12.2	4.86	82.8	23.8	2.4
$-\alpha^{3.7}/\alpha^{-5nt}\alpha$	1	12.0	6.02	65.9	19.9	2.8
$-\alpha^{3.7}/-\alpha^{3.7}$	2	11.9	5.55	78.7	21.5	2.3
$\alpha^{PA-1}\alpha/\alpha^{-5nt}\alpha$	1	11.1	6.11	71.0	18.2	1.0
$-\alpha^{3.7}/\alpha^{PA-1}\alpha$	4	10.1	5.45	72.4	18.5	1.8
$\alpha^{PA-1}\alpha/\alpha^{PA-1}\alpha$	13	9.8	5.18	66.5	17.3	1.8

^a . Accurate Hb H levels were unfortunately not obtained.

FIG. 4c/1. Pedigrees of two families with several members with Hb H disease. The presence of Hb H was detected by electrophoresis and was confirmed by chromatography; its quantity, however, could not be determined because of the age of the samples.

β -Thal. A total of 123 β -thal chromosomes were analysed; 40 β -thal major, six β -thal intermedia, 25 β -thal traits (parents and/or siblings), and six compound heterozygotes (four with Hb S- β -thal, two with Hb E- β -thal). The patients came from 46 unrelated families; 38 (82.6%) were Kuwaiti Arabs, three (6.5%) were Bedouin, two (4.3%) were Indian, and one (2.2%) each was of Lebanese, Egyptian, and Palestinian nationality. The origins of the 38 Kuwaiti families were: 20 (52.6%) from Saudi Arabia, nine (23.7%) from Iran, and six (15.8%) from Iraq, and in three (7.9%) the ancestry could not be ascertained. The age range of the 40 transfusion-dependent patients (20 males, 20 females) was 1-26 years. Those with thalassaemia intermedia ranged in age from 8 to 17 years.

As many as 12 β -thal alleles were identified; six Mediterranean alleles [IVS-II-1 (G $\rightarrow$ A), CD 39 (C $\rightarrow$ T), IVS-I-6 (T $\rightarrow$ C), IVS-I-110 (G $\rightarrow$ A) CD 8 (-AA), and IVS-I-1 (G $\rightarrow$ A)] were present in 79 (64.2%) of all β -thal chromosomes studied. Four Asian Indian mutations [IVS-I-5 (G $\rightarrow$ C), IVS-I 3' end 25 nt deletion, CDs 8/9 (+G), and the 619 bp deletion] were present in 31 (25.2%), and two Kurdish/Iranian alleles [CDs 36/37 (-T) and CD 44 (-C)] were found in 13 (10.6%). Among the thalassaemia major and thalassaemia intermedia patients, 69.6% were homozygous for their β -thal alleles, while the others were compound heterozygotes.

Of the 96 β -thal chromosomes from the Kuwaiti Arabs, Mediterranean alleles were present in 56 (58.3%), Asian Indian alleles in 28 (29.2%), and Kurdish/Iranian alleles in 12 (12.5%). However, the IVS-I 3' end 25 nt deletion was found only among Kuwaitis of Iranian origin, while the IVS-II-1 (G $\rightarrow$ A), IVS-I-6 (T $\rightarrow$ C), and IVS-I-1 (G $\rightarrow$ A) alleles were seen mostly in Kuwaitis of Saudi Arabian ancestry. The IVS-I-110 (G $\rightarrow$ A) mutation was absent in Kuwaitis, and the CD 39 (C $\rightarrow$ T) had a low prevalence (Table 4c/2).

Three of the six patients with thalassaemia intermedia were homozygous for the IVS-II-1 (G $\rightarrow$ A) mutation and one for the IVS-I-6 (T $\rightarrow$ C) mutation, while two were compound heterozygotes for the CD 8 (-AA) and the IVS-II-1 (G $\rightarrow$ A) mutations.

SS. There were 16 patients from 14 unrelated families; all were Kuwaiti Arabs except for one family each from Bahrain, Jordan, and Yemen, and one was a Bedouin. The patients (10 males, six females) were aged 2-12 years, except for two adults aged 24 and 33 years, respectively. The total Hb level ranged from 8.1 to 11.3 g/dl with a mean of 10.1 ± 1.0 g/dl. All had high Hb F levels (16-30.2%), except three patients (the Yemeni, the Jordanian, and the Bedouin) who had 5.4, 4.8, and 0.4%, respectively.

Haplotyping of the β^S chromosomes was carried out in eight SS patients with high Hb F levels and in one with low Hb F. The latter was the Jordanian patient who was homozygous for haplotype #19 (Benin), while seven of the eight patients with high Hb F were homozygous for haplotype #31 (Saudi Arabia/India). A 5-year-old Kuwaiti girl with a Hb F level of 30.2% and G_{γ} of 64.4% was heterozygous for haplotype #19 and a hybrid chromosome with a C at -158 of the G_{γ} promoter, which is characteristic of haplotype #19. However, in the LCR-HS-2, the haplotype-specific

point mutations (A at -10905, T at -10924, and A at -10390) were characteristic of haplotype #31 (17).

Interaction of α -Thal and Sickle Cell Trait. Twenty-four AS individuals had their α -globin gene patterns determined. As shown in Fig. 4c/2, there is an inverse relationship between the Hb S concentration and number of α -globin genes. The lowest levels were observed in individuals with a homozygosity for the poly A mutation; the average of 18.9% is only slightly higher than the 16.7% found for a Hb S heterozygote with Hb H disease due to a poly A mutation and a deletional α -thal-1 ($\alpha^{\text{PA-1}}\alpha/--$) (26)

DISCUSSION

This study was limited to patients attending three of the six major hospitals in Kuwait which handle the majority of the paediatric haematology cases in the country. Thus, the data described here provide a fair representation of the spectrum of major haemoglobinopathies in Kuwait.

α -Thal is the most prevalent trait in the Arabian Gulf and the highest prevalence (60%) is among the Mutair tribe of Saudi Arabia (3). Molecular characterisation of α -thal determinants in the region has only been reported from Saudi Arabia (20,21). The - α (3.7 kb) deletion is the most prevalent, and the - α (4.2 kb) deletion is also seen. Since the α -thal-1 ($--/\alpha\alpha$) trait is uncommon in the Gulf, one would expect Hb H disease to be rare in Kuwait as has been reported for Saudi Arabia (22). The present study, however, has shown that Hb H disease is common with seven unrelated, non-consanguineous families seen in a 3-month period. All cases were due to either homozygosity for the poly A (AATAAA $\rightarrow$ AATAAG) mutation or its co-existence with the - α (3.7 kb) or the IVS-I pentanucleotide ($\alpha^{-5\text{nt}}\alpha$) deletions.

The clinical picture of Hb H disease in our patients is quite variable. Although none were transfusion-dependent, a few have been frequently transfused. One 10-year-old had cholelithiasis and has had a cholecystectomy, and one 13-year-old had associated chronic ulcerative colitis which contributed to his anemia. Two apparently healthy parents (two males) who had not been diagnosed before were found to be homozygous for the poly A mutation. Their homozygous children were more severely affected. The reason for this degree of variability is not clear.

The poly A (AATAAA $\rightarrow$ AATAAG) mutation was first described in the Eastern Oasis of Saudi Arabia (23) and has been reported from other Mediterranean populations including Italy, Greece, Turkey, and the former Yugoslavia (24-26). Its frequency in the Gulf States has not been documented. The other nondeletional α -thal mutations reported from Mediterranean populations include the initiation CD mutation (ATG $\rightarrow$ ACG), the termination CD mutation (Hb Icaria), and the IVS-I donor splice site pentanucleotide deletion ($\alpha^{-5\text{nt}}$) (27,28). No initiation or termination CD mutations were detected in the present study but two chromosomes from one of the families (Fig. 4c/1) studied carried the pentanucleotide deletion. This deletion (GGTGA) removes the *Hph* I site in the splice junction of the IVS-I of the $\alpha 2$ -globin gene (29) and a few cases have been reported from Italy and Greece (25,27).

FIG. 4c/2. The levels of Hb S in Hb S heterozygotes with different α -globin gene deficiencies as indicated.

The presence of various β -thal alleles confirms the heterogeneity of the Kuwaiti population. Mediterranean alleles predominate, followed by mutations common among East Indians. This demonstrates the long-existing influence of these two regions on populations of the Arabian Gulf. The predominant allele in the United Arab Emirates is the IVS-I-5 (G $\rightarrow$ C) mutation, accounting for 60% (30), in Iran the IVS-II-1 (G $\rightarrow$ A) mutation accounts for 21.9%, and the IVS-I-5 (G $\rightarrow$ C) mutation for 12.5% (31), while the IVS-I-110 (G $\rightarrow$ A) and the IVS-I-5 (G $\rightarrow$ C) mutations are the most prevalent alleles in Saudi Arabia (32).

Obviously, the high rate of consanguineous marriages explains the high prevalence of homozygosity even for the rare alleles. It is interesting that the IVS-I-110 (G $\rightarrow$ A) mutation was not found among Kuwaiti Arabs (including those who originated from Saudi Arabia), but the rare mutation (IVS-I 3' end 25 nt deletion) was found in three unrelated families (two homozygotes, 3 heterozygotes). Its prevalence (8%) is identical to that reported among United Arab Emirates nationals (3), while the figure for Iran is 1.2% (31).

The β^S haplotype is similar to that found in Eastern Saudi Arabia, namely haplotype #31 (33). The one case with a homozygosity for haplotype #19 was a Jordanian patient. It is interesting that one 5-year-old SS patient with 30.2% Hb F and 64.4% G_γ was found to be a compound heterozygote for haplotype #19 and an atypical hybrid chromosome. This hybrid probably arose from crossover events placing sequences characteristic of the HS-2 of haplotype #31 in juxtaposition to the 5' flanking region of haplotype #19. Similar hybrid β^S chromosomes have been reported from Turkey and Nigeria (17,18).

While there is an apparent homogeneity in the β^S haplotypes and Hb H determinants within the Kuwaiti population, there is considerable heterogeneity of the prevalent β -thal alleles. However, if the individuals are grouped according to their ancestral origins, the number of prevalent alleles in each group decreases. The Bedouins appear to have a quite different pattern of β -thal alleles compared to other Kuwaiti Arabs. Also, the single Bedouin SS patient in the study had a low Hb F (0.4%) value, unlike the Kuwaiti Arab patients with Hb F values of 20%; unfortunately, haplotyping was not done in the former. It will be interesting to see if our continued studies, involving larger numbers, will bear out these differences. The present findings, however, have significant implications for genetic counselling and any proposed prenatal diagnosis program in Kuwait.

Acknowledgements. This study was supported by the United States Public Health Services research grant HLB-05168.

REFERENCES

1. Abu-Hakima, A.M.: *The Modern History of Kuwait*, page 1, Luzac & Co. Ltd., London, 1983.

2. White, J.M., Byrne, M., Richards, R., Buchanan, T., Katsoulis, E., and Weerashing, K.: Red cell genetic abnormalities in Peninsular Arabs; sickle hemoglobin, G6PD deficiency and α - and β -thalassemia. *J. Med. Genet.*, 23: 245, 1986.
3. Ganesaru, K., Acquaye, J., Samuel A.P.W., Sejeny, S.A., and Omer, A.: Intertribal variation in the prevalence of α -thalassemia in Saudi Arabia. *Birth Defects*, 23:365, 1988.
4. El-Hazmi, M.A.F.: Haemoglobin disorders: A pattern of thalassaemia and haemoglobinopathies in Arabia. *Acta Haematol.*, 68:43, 1982.
5. Righetti, P.G., Gianazza, E., Bianchi-Bosisio, A., and Cossu, G.: Conventional isoelectric focusing and immobilized pH gradients for hemoglobin separation and identification. In: *The Hemoglobinopathies*, edited by T.H.J. Huisman, *Methods in Hematology*, Vol. 15, page 47, Churchill Livingstone, Edinburgh, 1986.
6. Bissé, E. and Wieland, H.: High-performance liquid chromatographic separation of human haemoglobins. Simultaneous quantitation of foetal and glycated haemoglobins. *J. Chromatogr.*, 434:95, 1988.
7. Kutlar, A., Kutlar, F., Gu, L-G., Mayson, S.M., and Huisman, T.H.J.: Fetal hemoglobin in normal adults and β -thalassemia heterozygotes. *Hum. Genet.*, 85:106, 1990.
8. Huisman, T.H.J. and Jonxis, J.H.P.: *The Hemoglobinopathies Techniques of Identification*, Clinical and Biochemical Analysis, Vol. 6, Marcel Dekker, Inc., New York, 1977.
9. Shelton, J.B., Shelton, J.R., and Schroeder, W.A.: High performance liquid chromatographic separation of globin chains on a large-pore C₄ column. *J. Liq. Chromatogr.*, 7:1969, 1984.
10. Kutlar, F., Kutlar, A., and Huisman, T.H.J.: Separation of normal and abnormal hemoglobin chains by reversed-phase high-performance liquid chromatography. *J. Chromatogr.*, 357:147, 1986.
11. Poncz, M., Solowiejczyk, D., Harpel, B., Mory, Y., Schwartz, E., and Surrey, S.: Construction of human gene libraries from small amounts of peripheral blood: Analysis of β -like globin genes. *Hemoglobin*, 6:27, 1982.
12. Çürük, M.A., Yüregir, G.T., Asadov, C.D., Dadasova, T., Gu, L-H., Baysal, E., Gu, Y-C., Ribeiro, M.L.S., and Huisman, T.H.J.: Molecular characterization of β -thalassemia in Azerbaijan. *Hum. Genet.*, 90:417, 1992.
13. Boletini, E., Svobodova, M., Divoky, V., Baysal, E., Çürük, M.A., Dimovski, A.J., Liang, R., Adekile, A.D., and Huisman, T.H.J.: Sickle cell anemia, sickle cell β -thalassemia, and thalassemia major in Albania: Characterization of mutations. *Hum. Genet.*, 93:182, 1993.
14. Ringelhann, B., Szelenyi, J.G., Horanyi, M., Svobodova, M., Divoky, V., Indrak, K., Hollán, S., Marosi, A., Laub, M., and Huisman, T.H.J.: Molecular characterization of β -thalassemia in Hungary. *Hum. Genet.*, 92:385, 1993.
15. Sanger, F., Nicklen, S., and Coulson, A.R.: DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*, 74:5463, 1977.
16. Lanclos, K.D., Öner, C., Dimovski, A.J., Gu, Y-C., and Huisman, T.H.J.: Sequence variations in the 5' flanking and IVS-II regions of the G_γ - and A_γ -globin genes of β^S chromosomes with five different haplotypes. *Blood*, 77: 2488, 1991.

17. Öner, C., Dimovski, A.J., Altay, Ç., Gürgey, A., Gu, Y-C., Huisman, T.H.J., and Lanclos, K.D.: Sequence variations in the 5' hypersensitive site-2 of the locus control region of β^S chromosomes are associated with different levels of fetal globin in Hemoglobin S homozygotes. *Blood*, 79:813, 1992.
18. Adekile, A.D., Kitundu, M.N., Liu, J-S., Gu, L-H., Adeodu, O.O., Lanclos, K.D., and Huisman, T.H.J.: Haplotypes in SS patients from Nigeria; characterization of one atypical β^S haplotype no. 19 (Benin) associated with elevated Hb F and high G_γ levels. *Ann. Hematol.*, 65:41, 1992.
19. Baysal, E. and Huisman, T.H.J.: Detection of common deletional α -thalassaemia-2 determinants by PCR. *Am. J. Hematol.*, 46:208, 1994.
20. El-Hazmi, M.A.F.: Leftward deletion of α -thalassaemia in the Saudi Arabian population. *Hum. Genet.*, 74:219, 1986.
21. El-Hazmi, M.A.F.: α -Thalassaemia in Saudi Arabia: Deletion pattern. *Hum. Genet.*, 76:196, 1987.
22. El-Hazmi, M.A.F.: Haemoglobinopathies, thalassaemias, and enzymopathies in Saudi Arabia. *Acta Haematol.*, 78:130, 1987.
23. Pressley, L., Higgs, D.R., Clegg, J.B., Perrine, R.P., and Weatherall, D.J.: A new genetic basis for Hb H disease. *N. Engl. J. Med.*, 303:1383, 1980.
24. Thein, S.L., Wallace, R.B., Pressley, L., Clegg, J.B., Weatherall, D.J., and Clegg, J.B.: The polyadenylation site mutation in the α -globin gene cluster. *Blood*, 71:313, 1988.
25. Galanello, R., Aru, B., Dessi, C., Addis, M., Paglietti, E., Melis, M.A., Cocco, S., Massa, P., Giagu, N., Barella, S., Turco, M.P., Maccioni, L., and Cao, A.: Hb H in Sardinia: Molecular, hematological and clinical aspects. *Acta Haematol.*, 88:1, 1992.
26. Yüregir, G.T., Aksoy, K., Çürük, M.A., Dikmen, N., Fei, Y-J., Baysal, E., and Huisman, T.H.J.: Hb H disease in a Turkish family resulting from the interaction of a deletional α -thalassaemia-1 and a newly discovered poly A mutation. *Br. J. Haematol.*, 80:527, 1992.
27. Traeger-Synodinos, J., Kanavakis, E., Tzetis, M., Kattamis, A., and Kattamis, C.: Characterization of non-deletion α -thalassaemia mutation in the Greek population. *Am. J. Hematol.*, 44:162, 1993.
28. Higgs, D.R., Vickers, M.A., Wilkie, A.O.M., Pretorius, I-M., Jarman, A.P., and Weatherall, D.J.: A review of the molecular genetics of the human α -globin gene cluster. *Blood*, 73:1081, 1989.
29. Orkin, S.H., Goff, S.C., and Hechtman, R.L.: Mutation in an intervening sequence splice junction in man. *Proc. Natl. Acad. Sci. USA*, 78:5041, 1981.
30. Quaife, R., Al-Gazali, L., Abbes, S., Fitzgerald, P., Fitches, A., Valler, D., and Old, J.M.: The spectrum of β thalassaemia mutations in the UAE national population. *J. Med. Genet.*, 31:59, 1994.
31. Vernavides, L., Old, J., Mitchell, M., Miller, L., Fitches, A., Valler, D., Karimi-Nejad, R., Layton, M., and Karimi-Nejad, M.: Screening of β -thalassaemia mutations and application of prenatal diagnosis in the Iranian population. Abstract, Proceedings of the 5th International Conference on Thalassaemia and Haemoglobinopathies, Nicosia, Cyprus, 1993.
32. El-Hazmi, M.A.F., Al-Swailem, A.R., Wasy, A.S., and Al-Swailem, A.M.: Red cell disorders in Saudi Arabia. Abstract, Proceedings of the 5th International Conference on Thalassaemia and Haemoglobinopathies, Nicosia, Cyprus, 1993.

33. Padmos, M.A., Roberts, G.T., Sackey, K., Kulozik, A., Bail, S., Morris, J.S., Serjeant, B.E., and Serjeant, G.T.: Two different forms of homozygous sickle cell disease occur in Saudi Arabia. *Br. J. Haematol.*, 79:93, 1991.

CHAPTER 5

GENERAL DISCUSSION

CHAPTER 5

GENERAL DISCUSSION

The series of studies described in this dissertation highlight the differences in the molecular characteristics and in some of the clinical features of SS disease in two, ethnically and geographically, distinct people. The studies amply illustrate the influence of genetic (linked or unlinked to the β^S mutation) and environmental factors on the phenotypic expression of the disease. The major areas of difference in the genetic factors are in the prevalent β^S haplotypes and frequency and pattern of co-existent α -thal trait, while the environmental factors include climate (especially the hot desert of Kuwait), and endemic infection (malaria in Nigeria).

β^S Haplotypes. There are five major haplotypes of the β^S -globin gene cluster that are numbered according to the scheme of Antonarakis et al (1), and also named for the parts of Africa and Asia where each is predominant. Thus, the Benin (#19) is prevalent in Central West Africa, the Bantu (#20) in Central and East Africa, the Senegal (#3) in Atlantic West Africa, the Cameroon (#17) among the Eton tribe of Cameroon, and Saudi Arabia/India (#31) in Eastern Saudi Arabia and the Indian sub-continent. There is evidence that each of the major haplotypes originated independently in the location where it is most predominant (2-5). However, the pattern of its introduction to other parts of the world is not always quite clear. The Benin haplotype appears to be the most widely distributed, being the type predominantly seen in Central West Africa, all over North Africa, in parts of the Middle East (Jordan, Iraq, and Israel), Southern and Eastern Europe (see Fig. 1b/2).

Among Nigerians (6), the Benin haplotype was seen in over 90% of chromosomes tested with a few haplotypes #3, #20, and #17. The latter were probably recently introduced from neighbouring communities and other parts of Africa. Among Kuwaitis (7) the predominant haplotype is #31, seen in over 80% of chromosomes with much lower frequencies for the Benin (12%) and Bantu (6%) haplotypes (Table 5/1). The Saudi Arabia/India haplotype probably originated in the Indus Valley during the Harappan civilisation (2,500 to 1,500 BC) and was subsequently introduced to other parts of the region with the dispersal of its people after the collapse of their civilisation and following the Aryan invasions (3,8).

The introduction of the Benin haplotype to North Africa, and subsequently to Southern Europe and the Middle East, was thought to be secondary to the trans-Saharan slave trade which flourished from the 9th century and reached its peak in the 14th century (9-11). However, several factors are against this hypothesis. One, there is a complete absence of other haplotypes (#20, #3, and #17) which should be present since slaves were also taken from areas of Africa where these haplotypes are prevalent. Secondly, many of the individuals in whom the β^S trait is seen are "white" with no recent evidence of African heritage in their pedigrees. Therefore, it has been postulated that the trait must indeed have been introduced to North Africa and South-

ern Europe early in pre-historic times, and that although the trait persists, morphological features of the Hb S carriers are now Caucasoid.

TABLE 5/1. Distribution of Haplotypes in 699 and 92 β^S Chromosomes From Nigerian and Kuwaiti Patients, Respectively

Haplotype	Nigerian	Kuwaiti
Benin (#19) %	93.0	12.0
Saudi Arabia/India (#31) %	0	80.4
Bantu (#20) %	1.0	6.5
Cameroon (#17) %	3.4	0
Senegal (#3) %	0.2	0
Atypical	2.4	0

Probably the earliest evidence of migration of the people of Central West Africa northwards was during the fertile era of the Sahara (between 10,000 and 2,000 BC) (9-12). At this period the Sahara was green with flowing rivers and it supported large populations of a Negroid people who lived, hunted, and tended a large variety of land and aquatic animals. This was before and during the Neolithic period when agriculture was introduced. However, with the onset of the irreversible desiccation of the Sahara, there was a massive outward migration that introduced people of Saharan heritage to West Africa, the Nile region, and North Africa. These significant early events probably accounted for the first introduction of the β^S gene to North Africa and subsequently to Southern Europe and the Middle East. If it is true that the mutation originally occurred on a chromosome with haplotype #19, it is evident how it became the most widely distributed of the different haplotypes.

It is interesting that one Bedouin family was encountered in Kuwait with β^S chromosomes bearing the Bantu (#20) haplotype mutation. This haplotype has only been described in people of East or Central African ancestry and has never been reported in an Arab. The ancestry of the semi-nomadic Bedouins of the Arabian Peninsula is not always clear, and this particular family could not satisfactorily indicate their lineage. The part of the peninsula with long-standing historical, trading, and cultural relationship with East Africa is the Sultanate of Oman (11). Their β^S haplotype pattern has not been reported; however, the severe clinical presentation and low Hb F levels in Omani SS patients indicate that they are not likely to carry the Saudi Arabia/India haplotype (13). How many of them will turn out to have the Bantu haplotype is still unknown.

In most societies studied, a few cases of atypical haplotypes have been described. Among Nigerian patients, about 3% of the β^S chromosomes carried atypical mutations, *i.e.* they could not be classified into any of the common haplotypes (6). No such atypicals have been found in Kuwait. However, in both populations there was one family each with SS patient(s) who were compound heterozygotes for a hybrid haplotype and a haplotype #19 chromosome. In both instances, the hybrid is a result of juxtaposition of haplotype #3- or #31-like sequences in the LCR HS-2 to

those of the flanking region of the G_γ promoter of a β^S chromosome with haplotype #19. These two are similar to a previously reported hybrid chromosome from a Turkish patient (Fig. 5/1) (14). These three patients illustrate the over-riding influence of the LCR HS-2 sequences on G_γ expression that, in spite of having haplotype #19-specific mutations in its 5' flanking promoter (*in cis* and *in trans*), all three have elevated Hb F and G_γ levels.

α -Thal Trait. α -Thal is probably the most common genetic trait worldwide, with a wider distribution than sickle cell trait. Most cases are due to deletions affecting varying portions of the two α -globin genes, with the $-\alpha(3.7\text{ kb})$ deletion (α -thal-2 trait) being the most common (15). However, a few nondeletional mutants causing α -thal have been characterised (16-19). In Nigeria, only the $-\alpha(3.7\text{ kb})$ deletion was seen, with a frequency of about 25% in both the SS and AS populations (20). It is interesting, however, that the distribution of the trait among the different ethnic groups in the country showed significant differences. The Ibos had the highest value (33%), while the Hausa and Yorubas had similar values (25 and 21%, respectively). The Fulanis had the lowest figure of about 0.04%. The latter are believed to be derived from Saharan Hamitic stock and share physiognomic features with Arabs (straight black hair, long pointed noses, etc.) and not much with Negroes. The frequency of the trait among Arabs of North Africa is about 0.04-0.18% (15). This supports the hypothesis of early human migrations across the Sahara (5).

Among Kuwaitis, on the other hand, the overall frequency of α -thal trait was 40%, but among the SS patients it was 31.5% and in the AS group 47% (7). The $-\alpha(3.7\text{ kb})$ deletion was found in 27.5% of the population and nondeletional α -thal in 13.5% (Table 5/2). The difference in the frequency of α -thal trait among Kuwaitis compared to Nigerians is significant ($\chi^2 = 5.1$, $p = 0.02$). Most of the difference is accounted for by the prevalence of nondeletional α -thal in the former, since the frequency of α -thal-2 trait is similar in both populations. Among the countries of the Arabian Gulf, the frequency of α -thal in the general population is similar in Kuwait (40%), Oman (38-45%), and Saudi Arabia (about 45% in the eastern oases), but significantly more than reported for Yemen (6.5%) and the United Arab Emirates (16.5%) (21,22). The poly A signal (AATAAA $\rightarrow$ AATAAG) mutation had previously only been reported from Saudi Arabia where Pressley et al (16) found a frequency of up to 20% in the Eastern Province. In Kuwait, the mutation is confined to people of Saudi ancestry and has not been seen in any other nationality. The other nondeletional α -thal seen among Kuwaitis is the $\alpha 2$ -globin gene 5' IVS-I pentanucleotide deletion that was first described in an Italian (18) and has also been reported from Greece (19), but not from any other country in the Arabian Peninsula. Interestingly, there is archeological evidence that Alexander the Great's army used Failaka, an island under Kuwait's sovereignty, as a camp in the 3rd century BC and may have introduced Mediterranean mutations to this locality (23).

Morbidity Patterns. It is well-established that SS patients with haplotype #31 or #3 have a considerably less severe course than their other counterparts. The reason for this has been attributed mainly to the higher levels of Hb F in such patients (24-26). It is, therefore, not surprising that severe physical growth retardation, skull bossing, gnathopathy, hand/foot syndrome, priapism, leg ulceration, acute chest syn-

FIG. 5/1. Hybrid haplotypes resulting from a crossover between chromosomes with haplotypes #3 (or #31) and #19. A is the Turkish patient (14), B is the Nigerian patient (6), and C is the Kuwaiti patient (35).

drome, severe fulminating infections, and other complications are not common among Kuwaiti patients. As reported for Eastern Saudi patients (27,28), the most common cause of acute illness is vaso-occlusive crisis, the incidence of which reaches its peak during the hottest month of the year, July, when the daily average temperature is about 45°C or 115°F. This indicates that climate probably plays an important role in triggering crisis in this region. Advice about staying out of the sun and liberal intake of fluids at this period of the year has proven to be quite useful in reducing the incidence of crisis in the summer months.

TABLE 5/2. Frequencies of Different α -Thal Determinants Among Nigerian and Kuwaiti Patients

Deletion	Nigerian (n = 284)	Kuwaiti (n = 60)
$-\alpha$ (-3.7 kb)	0.258	0.275
PA-1 (AATAAA~AATAAG)	0	0.102
IVS-I α ^{-5nt} α	0	0.033

It is interesting that the frequencies of splenomegaly in steady state patients from the two countries are similar (24 and 26% in Kuwait and Nigerian, respectively) (7,29). However, the major difference was in the size of the spleen palpated below the costal margin. Among Kuwaitis, the range of spleen size was 2-7 cm; the only patient with 13 cm had an acute sequestration crisis. Among Nigerian steady state patients, however, the range was 1-21 cm and more than half (61.8%) of those with splenomegaly had spleen ≥ 6 cm big. In comparison, the frequency of splenomegaly (1-5 cm) was 8% among American patients, who were mostly haplotype #19 like the Nigerian subjects (29). There is also considerable evidence relating splenomegaly to malaria in Nigerian patients. In particular, those with PGS are very similar to TSS which is recognised as a malaria hypersensitivity state. Moreover, Nigerian patients with PGS are more prone to anaemic crisis and some of them are at risk for hepatic cirrhosis. Table 5/3 compares some clinical features between Nigerian and Kuwaiti patients.

TABLE 5/3. Frequencies (%) of Some Clinical Features Among Nigerian and Kuwaiti Patients

	Nigerian	Kuwaiti
Spleen (enlarged)	26.0	24.0
Liver (enlarged)	52.0	15.2
Gallstones	4.8	15.2
Infections	37.5	19.0
Vaso-occlusive crisis	19.6	60.3
Haemolytic crisis	25.5	15.8

Padmos et al (27) found that splenomegaly was present in 30% of Western Saudi SS (haplotype #19) patients, but 53% in Eastern Saudi SS (haplotype #31) patients. One explanation for a higher frequency in the latter is, that because of less severe microvasculopathy, the infarctive process and eventual autosplenectomy are delayed. Among Nigerian patients, one factor that promotes splenomegaly is the proliferation of both the T and B cell systems that occurs with recurrent malaria (30). Regular malaria chemoprophylaxis significantly reduces the prevalence of splenomegaly in SS patients (31). It has also been shown that in Nigerian SS patients there is a direct relationship between the splenic size and the levels of serum anti-malaria antibody titres (29). Also, the high levels of serum immunoglobulins in the patients with gross splenomegaly is similar to the situation in TSS which is considered a malaria hypersensitivity state (31). Other clinical features associated with gross splenomegaly in Nigerian patients include hepatomegaly and digital clubbing. A previous study (31) demonstrated focal hepatic necrosis and fibrosis in some of these patients, but none showed overt cirrhosis.

The factor(s) that promote persistent splenic enlargement in some Kuwaiti patients has not been identified. In both Kuwaiti and Nigerian patients, there was no significant influence of α -thal on its pathogenesis (7,29). However, in both groups, splenomegaly was associated with relative hypersplenism with moderate reduction in blood cellular elements and a higher incidence of anaemic crisis (in the Nigerian group). There was one Kuwaiti patient with repeated transfusions who eventually required surgical splenectomy. The latter intervention is a last resort, especially since many of the patients with splenomegaly still retain their reticulo-endothelial function (7,29,32-34). There is also evidence that these enlarged spleens are actively producing immunoglobulins (Table 5/4).

Among Nigerian patients, there was evidence of preferential survival of SS patients with co-existent α -thal trait (2). The latter also had higher Hb levels, although a direct association with an overall less severe clinical course could not be demonstrated. In the Kuwaiti patients, on the other hand, there was significant association of co-existent α -thal trait with normal splenic function and absence of gallstones. Probably as a result of the improved spleen function, none of the patients with severe recurrent infections had co-existent α -thal trait. However, it is not clear whether other genetic and/or environmental factors protect Kuwaiti patients from severe bacterial infections. This is an area that deserved further research.

CONCLUSIONS

1. The predominant β^S haplotype among Nigerian patients is #19 (Benin), while among Kuwaitis it is haplotype #31 (Saudi Arabia/India). About 3% of Nigerians but no Kuwaiti had atypical haplotypes. One family each with a hybrid β^S chromosome was found in both groups.
2. The frequency of α -thal-2 trait is similar in both groups (25 and 27.5%, in Nigeria and Kuwait, respectively). However, about 13% of the latter, but none of the former, have nondeletional α -thal trait [poly A signal (AATAAA) $\rightarrow$

TABLE 5/4. Serum Immunoglobulins (mean $\pm$ SD) in Nigerian, American, and Kuwaiti SS Patients

Group	IgG (mg/dl)	IgM (mg/dl)	IgA (mg/dl)
<u>Nigerian</u>			
(n = 140)	2314.8 $\pm$ 100.1	562.9 $\pm$ 147.7	255.9 $\pm$ 14.6
Spleen 0 cm (n = 99)	1887.0 $\pm$ 112.2	283.0 $\pm$ 33.2	218.0 $\pm$ 16.1
Spleen $\leq$ 5 cm (n = 15)	2401.3 $\pm$ 193.7	407.6 $\pm$ 107.7	245.0 $\pm$ 37.2
Spleen 1-6 cm (n = 17)	3048.8 $\pm$ 254.8	885.1 $\pm$ 208.7	317.4 $\pm$ 37.4
Spleen $\geq$ 10 cm (n = 9)	2934.0 $\pm$ 417.6	1809.3 $\pm$ 1366.7	326.3 $\pm$ 67.5
<u>American</u>			
(n = 59)	1391.1 $\pm$ 56.6	173.5 $\pm$ 11.2	223.4 $\pm$ 12.7
Spleen 0 cm (n = 51)	1419.7 $\pm$ 64.1	166.4 $\pm$ 11.6	226.0 $\pm$ 14.0
Spleen $\leq$ 5 cm (n = 8)	1208.6 $\pm$ 58.0	218.6 $\pm$ 33.8	207.0 $\pm$ 30.5
<u>Kuwaiti</u>			
(n = 30) ^a	1440.8 $\pm$ 71.7	146.5 $\pm$ 12.3	229.2 $\pm$ 32.8
Spleen 0 cm (n = 19)	1433.5 $\pm$ 100.7	142.4 $\pm$ 15.8	222.7 $\pm$ 31.6
Spleen $\leq$ 5 cm (n = 6)	1433.3 $\pm$ 157.2	179.2 $\pm$ 28.7	268.8 $\pm$ 41.7
Spleen $\geq$ 5 cm (n = 5)	1476.0 $\pm$ 127.3	122.6 $\pm$ 24.1	207.6 $\pm$ 40.4

^a Unpublished data.

AATAAG) mutation and the $\alpha 2$ IVS-1 5' pentanucleotide (GAGGTGAGG→GAGG) deletion].

3. The commonest cause of hospitalisation among Kuwaitis is painful crisis, while among Nigerians it is bacterial or parasitic (malaria) infection.
4. Complications of SS disease, *e.g.* acute chest syndrome, skeletal abnormalities, leg ulceration, priapism, etc. are not common among Kuwaiti patients.
5. The prevalence of splenomegaly in steady state patients is similar in both populations (21.2 and 20.3% in Kuwait and Nigeria, respectively). However, when palpable, the spleen tends to be much larger in the latter.
6. There is indirect evidence that malaria plays a role in the pathogenesis of gross splenomegaly among Nigerian SS patients.
7. In both populations, splenomegaly is associated with relative hypersplenism, and among Nigerians there is increased evidence of anaemic crisis in those with large spleens.
8. Among Nigerians, patients with PGS also tend to have hepatomegaly and digital clubbing.
9. Splenomegaly is not related to co-existent α -thal in either group.
10. In about 60% of Kuwaiti patients the spleen is either well or partially visualised on labelled colloid scintigraphy.
11. Among Kuwaiti patients, co-existent α -thal trait is associated with good splenic function and the absence of gallstones.
12. The less severe clinical course among Kuwaiti patients is attributed to the pattern of β^S haplotype and higher frequency of α -thal trait compared to Nigerian patients.

REFERENCES

1. Antonarakis, S.E., Boehm, C.D., Serjeant, G.R., Theisen, C.E., Dover, G.J., and Kazazian, H.H., Jr.: Origin of the β^S globin gene in Blacks: The contribution of recurrent mutation or gene conversion or both. *Proc. Natl. Acad. Sci. USA*, 81:853, 1984.
2. Nagel, R.L. and Ranney, H.M.: Genetic epidemiology of structural mutations of the β globin gene. *Semin. Hematol.*, 27:342, 1990.
3. Nagel, R.L. and Fleming, A.F.: Genetic epidemiology of the β^S gene. In: *Epidemiology of Haematological Disease, Part II*, edited by A.F. Fleming, Bailliere's Clinical Haematology, Vol. 5, page 331, W.B. Saunders Company, London, 1992.

4. Flint, J., Harding, R.M., Boyce, A.J., and Clegg, J.B.: The population genetics of haemoglobinopathies. In: *Epidemiology of Haematological Disease, Part II*, edited by A.F. Fleming, Bailliere's Clinical Haematology, Vol. 5, page 215, W.B. Saunders Company, London, 1992.
5. Adekile, A.D.: Anthropology of the β^S gene-flow from West Africa to North Africa, the Mediterranean, and Southern Europe. *Hemoglobin*, 16:105, 1992.
6. Adekile, A.D., Kitundu, M.N., Gu, L-H., Adeodu, O.O., Lanclos, K.D., and Huisman, T.H.J.: Haplotypes in SS patients from Nigeria; characterization of one atypical β^S haplotype #19 (Benin) associated with elevated Hb F and high G_γ levels. *Ann. Hematol.*, 65:41, 1992.
7. Adekile, A.D. and Haider, M.Z.: Morbidity, β^S haplotype, and α -globin gene patterns among SS patients in Kuwait. *Acta Haematol.*, submitted.
8. Labie, D., Snirivas, R., Dunda, O., Dode, C., Lapoumeroulie, C., Devi, V., Devi, S., Ramasani, K., Elion, F., and Ducrocq, R.: Haplotypes in tribal Indians bearing the sickle gene: Evidence for the unicentric origin of the β^S mutation and the unicentric origin of the tribal populations of India. *Hum. Biol.*, 61:479, 1989.
9. Davidson, B.: *African Civilisation Revisited From Antiquity to Modern Times*, page 1, Africa World Press, Inc., Trenton, N.J., 1991.
10. Davidson, B.: *Africa in History*, page 1, Collier Books, New York, 1988.
11. Davidson, B.: *The Lost Cities of Africa*, page 7, Little, Brown and Company, Boston/Toronto, 1987.
12. Lhote, H.: *The World's Last Mysteries*, page 205, The Reader's Digest Association, Inc., Pleasantville, N.Y./Montreal, 1978.
13. White, J.M., Christie, B.S., Nam, D., Daar, S., and Higgs, D.R.: Frequency and clinical significance of erythrocyte abnormalities in Omanis. *J. Med. Genet.*, 30:396, 1993.
14. Oner, C., Dimovski, A.J., Altay, C., Gurgey, A., Gu, Y.C., Huisman, T.H.J., and Lanclos, K.D.: Sequence variations in the 5' hypersensitive site-2 of the locus control region of β^S chromosomes are associated with different levels of fetal globin in Hemoglobin S homozygotes. *Blood*, 79:813, 1992.
15. Higgs, D.R., Vickers, M.A., Wilkie, A.O.M., Pretorius, I-M., Jarman, A.P., and Weatherall, D.J.: A review of the molecular genetics of the human α -globin gene cluster. *Blood*, 73:1081, 1989.
16. Pressley, L., Higgs, D.R., Clegg, J.B., Perrine, R.P., and Weatherall, D.J.: A new genetic basis for Hb H disease. *N. Engl. J. Med.*, 303:1383, 1980.
17. Thein, S.L., Wallace, R.B., Pressley, L., Clegg, J.B., Weatherall, D.J., and Higgs, D.R.: The polyadenylation site mutation in the α -globin gene cluster. *Blood*, 71:313, 1988.
18. Orkin, S.H., Goff, S.C., and Hechtman, R.L.: Mutation in an intervening sequence splice junction in man. *Proc. Natl. Acad. Sci. USA*, 78:5041, 1981.
19. Galanello, R., Aru, B., Dessi, C., Addis, M., Paglietti, E., Melis, M.A., Cocco, S., Massa, P., Giagu, N., Barella, S., Turco, M.P., Maccioni, L., and Cao, A.: Hb H in Sardinia: Molecular, hematological and clinical aspects. *Acta Haematol.*, 88:1, 1992.
20. Adekile, A.D., Liu, J-C., Sulzer, A.J., and Huisman, T.H.J.: Frequency of the α -thalassaemia-2 gene among Nigerian SS patients and its influence on malaria titers. *Hemoglobin*, 17:73, 1993.

21. White, J.M., Byrne, M., Richards, R., Buchanan, T., Katsoulis, E., and Weerasinghi, K.: Red cell genetic abnormalities in Peninsular Arabs: Sickle haemoglobin, G6PD deficiency and α and β thalassaemia. *J. Med. Genet.*, 23:245, 1986.
22. El-Hazmi, M.A.F.: Haemoglobin disorders: A pattern for thalassaemia and haemoglobinopathies in Arabia. *Acta Haematol.*, 68:43, 1982.
23. *The Seals of Failaka*, Department of Antiquity and Museums, Kuwait National Museum, 1993.
24. Kulozik, A.E., Wainscoat, J.S., Serjeant, G.R., Kar, B.C., Al-Awamy, B., Esan, G.J.F., Falusi, A.G., Haque, S.K., Hilali, A.M., Kate, S., Ranasinghe, W.A.E.P., and Weatherall, D.J.: Geographical survey of the β^S globin gene haplotypes: Evidence for an independent Asian origin of the sickle cell mutation. *Am. J. Hum. Genet.*, 39:239, 1986.
25. Miller, B.A., Olivieri, N., Salameh, M., Ahmed, M., Antognetti, G., Huisman, T.H.J., Nathan, D.G., and Orkin, S.H.: Molecular analysis of the high-Hemoglobin F phenotype in Saudi Arabian sickle cell anemia. *N. Engl. J. Med.*, 316:244, 1987.
26. Powars, D.R.: Sickle cell anemia: β^S -gene cluster haplotype as prognostic indicators of vital organ damage. *Semin. Hematol.*, 28:202, 1991.
27. Padmos, M.A., Roberts, G.T., Kulozik, A., Bail, S., Morris, J.S., Serjeant, B.E., and Serjeant, G.R.: Two different forms of homozygous sickle cell disease occur in Saudi Arabia. *Br. J. Haematol.*, 79:93, 1991.
28. El-Hazmi, M.A.F.: Clinical manifestations and laboratory findings of sickle cell anaemia in association with α -thalassaemia in Saudi Arabia. *Acta Haematol.*, 74:155, 1985.
29. Adekile, A.D., McKie, K.M., Adeodu, O.O., Sulzer, A.J., McKie, V.C., Kutlar, F., Ramachandran, M., Kaine, W., Akenzua, G.I., Akolo, A.A., Asindi, A.A., Obinyan, E.A., Ogala, W.N., Ibrahim, M., and Huisman, T.H.J.: The spleen in sickle cell anemia: Comparative studies of Nigerian and U.S. patients. *Am. J. Hematol.*, 42:316, 1993.
30. Greenwood, B.M. and Vicki, R.M.: Evidence for a malaria mitogen in human malaria. *Nature*, 56:592, 1975.
31. Adekile, A.D., Adeodu, O.O., Jefe, A.O., and Adesanmi, W.O.: Persistent splenomegaly in sickle cell disease: Relationship to malaria. *Ann. Trop. Paediatr.*, 8:103, 1988.
32. Adeodu, O.O. and Adekile, A.D.: Clinical and laboratory observations on sickle cell patients with gross splenomegaly. *Acta Paediatr. Scand.*, 79:687, 1990.
33. Adekile, A.D., Adeodu, O.O., Reindorf, C.A., Johnson, W., and Dairo, B.A.: Pitted red cell counts in Nigerian children with sickle cell anemia: Relationship to splenic size and age. *J. Natl. Med. Assoc.*, 83:329, 1991.
34. Adekile, A.D., Tuli, M., Haider, M.Z., Al-Zaabi, K., Mohammadi, S., and Owunwanne, A.: Influence of α -thalassaemia trait on spleen function in sickle cell anemia patients with high Hb F. *Am. J. Hematol.*, in press.
35. Adekile, A.D., Gu, L-H., Baysal, E., Haider, M.Z., Al-Fuzae, L., Aboobacker, K.C., Al-Rashied, A., and Huisman, T.H.J.: Molecular characterization of α -thalassaemia determinants, β -thalassaemia alleles, and β^S haplotypes among Kuwaiti Arabs. *Acta Haematol.*, 92:176, 1994.

CHAPTER 6

SUMMARY

CHAPTER 6

SUMMARY

The main objective of this dissertation is to compare the clinical features and patterns of morbidity of Nigerian and Kuwaiti SS patients and to identify possible factors that influence the observed patterns. Apart from identifying the common causes of hospitalisation, and defining clinical features of steady state patients, the spleen was used as an index organ to study the pathophysiology of the disease. The major differentiating molecular factors examined were β^S haplotypes and frequency of co-existent α -thal trait.

A review of the relevant literature is presented in **Chapter 1b**. It gives a brief history of SS disease, laboratory diagnosis, pathophysiology, clinical features and management. The factors which influence its clinical severity were discussed in detail. These include co-existent α -thal, Hb F levels, RBC enzyme deficiencies (e.g. G6PD) and environmental factors. The factors influencing Hb F levels in SS disease were also discussed in detail and they include age, gender, α -thal, mutations in the promoter regions of the G_γ - and A_γ -globin genes, mutations in the β -globin gene LCR and the promoter region.

Chapter 2 gives details about the patients and the methodology used in the different studies reported.

Chapter 3 is a collection of clinical and molecular studies carried out on Nigerian patients.

Chapter 3a is adapted from two papers which describe our original observations on the spleen in Nigerian SS patients. The chapter identifies the frequency of splenomegaly in a group of 139 SS patients and focuses on the clinical and laboratory parameters in those with PGS, i.e. aged above 8 years, with a spleen of ≥ 10 cm below the costal margin and which had been palpable for at least 6 months. The findings were compared to those in a group of SS patients without palpable spleens matched for age, sex and social class. The former had lower levels of blood cellular elements and Hb (probably secondary to hypersplenism) and increased levels of serum bilirubin. They also had more frequent hepatomegaly which was associated with digital clubbing in some. Retrospective analysis of hospital admissions showed that gross splenomegaly is associated with frequent anaemic crisis. Moreover, the PGS group had significantly higher levels of serum IgM, and hepatic histopathology showed moderate-to-severe sinusoidal dilatation and portal lymphocytic infiltration while a few showed evidence of new fibrous tissue formation and basement membrane degeneration. There was also uniform response to prolonged proguanil therapy. All these findings suggest that PGS is similar to TSS, which is a malaria hypersensitivity state and that some have hepatic pre-cirrhotic pathological features.

Chapter 3b is a comparative study of the spleen among Nigerian and American SS patients. Since there had been anecdotal reports attributing splenomegaly in SS disease to the effects of malaria, the study was designed to compare patients in a malaria endemic region (Nigeria) to those in a malaria free zone (America). It was found that in steady state patients, the frequency of splenomegaly was 23% in the former and 8% in the latter. There was a linear relationship between the spleen size and serum immunoglobulins among Nigerians. In both groups there was evidence of hypersplenism in those with splenomegaly. Reticulo-endothelial function (from circulating pitted red cells) appeared to be intact and even enhanced in those with large spleens. Among the Nigerians in the study, there was an association between spleen size and the level of anti-malaria antibody levels. In both populations, co-existent α -thal did not appear to influence splenomegaly. The study provided further support for the influence of malaria on the pathogenesis of splenomegaly in Nigerian SS patients.

Chapter 3c. This paper describes the frequency of α -thal-2 trait which is the only type of α -thal seen in Nigeria, among a large group of SS patients ($n = 284$) and their AS and AA relatives ($n = 62$) drawn from the different tribes of Nigeria. The frequency was found to be about 25% in both SS and controls. However, there were significant differences in the frequencies among the tribes. It was highest among Ibos (33%) and lowest among Fulanis (0.04%). Among the SS group, α -thal trait was associated with increased survival and higher Hb levels. Patients with α -thal trait had lower levels of some anti-malaria antibody titres, suggesting less intense immune stimulation probably secondary to lower parasite rates.

Chapter 3d. This paper describes the pattern of β^S -globin gene cluster haplotypes in over 600 chromosomes from Nigerian SS patients and their relations. Haplotype #19 was the most prevalent, seen in 93% of chromosomes, while #17 was seen in 3.4%, and #20 in 1%, and 2.4% represented atypicals. There were no observed differences among the different tribes within the country. There was one family in whom several members carried a hybrid chromosome with haplotype #19 characteristics juxtaposed to haplotype #3 characteristics. The details of this hybrid are provided and are similar to chromosomes described from Turkey and Kuwait.

Chapter 4. These chapters contain studies carried out on Kuwaiti patients.

Chapter 4a describes the common clinical features and the causes of acute illness among Kuwaiti SS patients. It also documents the prevalent β^S haplotypes and α -thal patterns among them. Complications of the disease, *e.g.* acute chest syndrome, leg ulceration, severe fulminant infections, etc., are not common. Vaso-occlusive crisis is the most common cause of acute illness. Splenomegaly occurs in about 21.2%, hepatomegaly and gallstones in 15.2% each. The predominant haplotype is the Saudi Arabia/India haplotype #31, with a few cases of Benin and Bantu haplotypes. The frequency of α -thal trait in the SS and AS individuals is about 40%, with the $-\alpha(3.7 \text{ kb})$ deletion occurring in 27.5% and nondeletional α -thal in about 13%.

Chapter 4b. Spleen function was assessed in a group of 20 Kuwaiti patients using ^{99m}Tc -labeled tin colloid uptake. Seven (35%) had normal visualisation, while the spleen was partially demonstrable in 25%, and not seen at all in 40%. In the group

with normal uptake, the frequency of α -thal trait was 57.1%, while in the group with no function, it was 18.8%, thus demonstrating the positive association between co-existent α -thal and normal spleen function.

Chapter 4c. This paper describes the molecular characteristics of the major haemoglobinopathies in Kuwait and underscores the genetic heterogeneity of the population. Three mutant α -thal alleles were identified: $-\alpha(3.7 \text{ kb})$ deletion, the poly A signal (AATAAA $\rightarrow$ AATAAG) mutation, and the $\alpha 2$ IVS-I 5' splice junction pentanucleotide (GAGGTGAGG $\rightarrow$ GAGG) deletion. Twelve different β -thal alleles were characterised, the commonest being Mediterranean alleles. The two β^S haplotypes encountered were the Saudi Arabian (#31) and the Benin (#19).

Chapter 5. This chapter gives an overall discussion of all the studies reported and the conclusions reached. The possible modes of introduction of the observed mutants (β^S haplotypes #31, #19, and #20, and deletional and nondeletional α -thal) to Kuwait and Nigeria are discussed. The major conclusion is that the less severe clinical course among Kuwaitis is related to the β^S haplotype with consequent elevated Hb F and the higher frequency of co-existent α -thal trait.

In summary, the data presented in this dissertation show that sickle cell anaemia is a milder disease among Kuwaitis than Nigerians. However, the frequency of splenomegaly in steady state is similar in both groups but when enlarged, the spleen tends to be much larger in Nigerian patients. The predominant β^S haplotype among Nigerians is the Benin (#19), while among Kuwaitis it is the Saudi Arabia/India (#31). While the frequency of α -thal-2 (3.7 kb deletion) trait is similar in both groups (about 25%), nondeletional α -thal is found in an additional $\sim 13\%$ of Kuwaiti patients. In the latter, α -thal trait is associated with normal spleen function and absence of severe recurrent infections and gallstones. The same could not be documented for Nigerians. It therefore appears that the milder disease among Kuwaitis is a function of both the Saudi Arabia/India haplotype and the higher frequency of α -thal determinants.

SAMENVATTING

SAMENVATTING

De belangrijkste doelstelling van dit proefschrift was de vergelijking van de klinische verschijnselen en morbiditeitspatronen van patiënten die homozygoot zijn voor de sikkelcelmutatie in Nigeria en Kuwait.

Vervolgens was het de bedoeling de factoren te identificeren die een invloed hebben op de waargenomen patronen. Naast de onderkenning van de belangrijkste oorzaken van hospitalisatie en de definitie van klinische verschijnselen van patiënten in een stabiele situatie, werd de milt gebruikt als een index orgaan voor de bestudering van de pathofysiologie van de ziekte. De belangrijkste onderscheidende moleculaire factoren die onderzocht werden, waren β^s haplotypen en de frequentie van tegelijk aanwezige heterozygotie voor alpha-thalassemie.

Een overzicht van de relevante literatuur is gepresenteerd in hoofdstuk 1b. Het geeft een korte historie van sikkelcelanemie, laboratorium diagnostiek, pathofysiologie, klinische verschijnselen en management. De factoren die de klinische ernst beïnvloeden, worden in detail besproken. Deze betreffen onder meer tegelijk aanwezig alpha-thal, HbF niveaus, erythrocyt enzym deficiënties (b.v. G6PD) en omgevingsfactoren. De factoren die de HbF niveaus bij sikkelcelanemie beïnvloeden, zijn leeftijd, alpha-thal, mutaties in de promotor regio's van de γ - en β -globine genen, mutaties in het β -globine gen LCR en de promotor.

Hoofdstuk 2 gaat in op de patiënten en de methodologie die in de verschillende studies gebruikt is.

Hoofdstuk 3 is een verzameling van klinische en moleculaire onderzoeken uitgevoerd bij Nigeriaanse patiënten.

Hoofdstuk 3a betreft onderzoek met betrekking tot de milt bij Nigeriaanse sikkelcelanemie patiënten. Naast de frequentie van splenomegalie bij 139 patiënten komen in dit hoofdstuk de klinische en laboratoriumparameters in de patiënten met PGS, d.w.z. boven de leeftijd van 8 jaar, met een milt van ≥ 10 cm onder de ribbenboog en die palpabel was voor meer dan 6 maanden. De bevindingen werden vergeleken met die in een groep van sikkelcelanemie patiënten zonder palpabele milt van dezelfde leeftijd, geslacht en sociale status. De eerste groep had lagere aantallen bloedcellen en Hb en verhoogde serum bilirubine waarden. Ze hadden ook vaker hepatosplenomegalie die bij sommigen samenhang met onregelmatige verdikkingen van de vingergewrichten.

Retrospectief onderzoek toonde aan dat ernstige splenomegalie is geassocieerd met frequente anemische crises. Bovendien had de PGS groep significant hogere serum IgM waarden en de leverhistopathologie liet matig tot ernstige sinusoidale dilatatie en portale lymfocyttaire infiltratie zien, terwijl enkelen tekenen vertoonden van nieuwe bindweefselvorming en basaal membraan degeneratie. De respons op langdurige proguanil therapie was identiek. Alle bevindingen doen vermoeden dat PGS gelijk is aan TSS, hetgeen een malaria overgevoeligheid is en dat sommigen pre-cirrhotische leverafwijkingen hebben.

Hoofdstuk 3b is een vergelijkende studie van de milt bij sikkelcelanemie patiënten uit Nigeria en Kuwait. Aangezien splenomegalie bij sikkelcelanemie in enkele mededelingen aan de effecten van malaria is toegeschreven, werd het onderzoek zo opgezet dat een

vergelijking plaatsvond tussen patiënten in een malaria endemisch gebied (Nigeria) en een malaria vrij gebied (Amerika). Gevonden werd dat bij stabiele patiënten 23% van de eerste en 8% van de tweede groep splenomegalie vertoonde. Bij de Nigerianen werd een lineair verband gevonden tussen de grootte van de milt en de serum immunoglobuline waarden. In beide groepen werden aanwijzingen gevonden voor hypersplenie bij patiënten met splenomegalie. Reticulo-endotheliale functies bleken intact en zelfs versterkt bij patiënten met een grote milt. Bij de Nigerianen werd een verband vastgesteld tussen de grootte van de milt en de anti-malaria antilichaam titer. In beide populaties bleek tegelijk aanwezig alpha-thal niet van invloed te zijn op de splenomegalie. Het onderzoek maakte een invloed van malaria op het ontstaan van splenomegalie bij sikkelcelanemie patiënten aannemelijk.

Hoofdstuk 3c beschrijft de frequentie van alpha-thal-2, hetgeen het enige alpha-thalassemie type is dat gevonden wordt in Nigeria, bij 284 patiënten met sikkelcelanemie en hun heterozygote en homozygoot normale verwanten (n=62) afkomstig van verschillende Nigeriaanse stammen. Zowel bij de sikkelcelanemie patiënten als de controles was de frequentie ongeveer 25%. Er waren echter significante verschillen tussen de stammen. De Ibos vertoonden de hoogste frequentie (33%) en de Fulanis de laagste (4%). Bij sikkelcelanemie patiënten was alpha-thal-2 geassocieerd met een hogere levensverwachting en hogere Hb waarden. Patiënten met alpha-thal-2 hadden lagere waarden van enige anti-malaria antilichaam titers, hetgeen suggestief is voor een minder intense immuun stimulatie, mogelijk ten gevolge van lagere parasiet aantallen.

Hoofdstuk 3d. Hierin wordt de β^s -globine gencluster haplotypering van meer dan 600 chromosomen van Nigeriaanse sikkelcelanemie patiënten en hun verwanten beschreven. Haplotype 19 kwam het meeste voor, bij 93% van de chromosomen. Nr. 17 werd gevonden bij 3,4% en Nr. 20 bij 1%, terwijl bij de resterende 2,4% slechts atypische haplotypen voorkwamen. Er werden geen verschillen waargenomen tussen de verschillende stammen. In een familie werd bij meerdere familie-leden een hybride chromosoom gevonden dat gedeeltelijk bestond uit haplotype Nr. 19 naast Nr. 3 kenmerken. De details van deze hybride zijn beschreven en komen overeen met die beschreven zijn bij patiënten uit Turkije en Kuwait.

Hoofdstuk 4. Hierin worden de onderzoeken bij patiënten uit Kuwait beschreven.

Hoofdstuk 4a geeft de meest voorkomende klinische kenmerken en de oorzaken van acute problemen bij sikkelcelanemie patiënten. Het beschrijft ook de β^s haplotypen en alpha-thal patronen. Complicaties van de ziekte kwamen niet vaak voor. Vaso-occlusieve crisis is de meest voorkomende oorzaak van acute ziekte. Splenomegalie komt voor bij 21,2%, hepatomegalie en galstenen elk bij 15,2%. Het meest voorkomende haplotype is het Saudi Arabische/indische haplotype Nr. 31, met daarnaast enkele Benin en Banke haplotypen. De frequentie van alpha-thal bij homozygote en heterozygote sikkelcelanemie is ongeveer 40%. De -alpha(3,7kb) deletie wordt gevonden bij 27,5% terwijl ongeveer 13% geen deletie vertoont.

Hoofdstuk 4b. De miltfunctie werd onderzocht in een groep van 20 patiënten uit Kuwait met behulp van ^{99m}Tc -gelabeld tin colloïd opname. Zeven (35%) keer was de milt normaal aantoonbaar, in 25% partieel en in 40% in het geheel niet zichtbaar. In de groep met

normale opname was de frequentie van alpha-thal 57,1% terwijl in de groep zonder functie het percentage 18,8% was. Dit toont de positieve associatie tussen gelijktijdig voorkomend alpha-thal en een normale miltfunctie aan.

Hoofdstuk 4c. Hierin worden de moleculaire kenmerken van de belangrijkste hemoglobi-nopatieën in Kuwait beschreven en onderstreept de genetische heterogeniteit van de populatie. Drie mutante alpha-thal allelen werden geïdentificeerd: -alpha(3,7kb) deletie, de poly A signaal (AATAAA → AATAAG) mutatie en de alpha2IVS-I 5' splice junction pentanucleotide (GAGGTGAGG → GAGG) deletie. Twaalf verschillende β -thal allelen werden gekarakteriseerd, de meest voorkomende waren Mediterrane allelen. De twee β^s haplotypen die gevonden werden, waren het Saudi Arabische (Nr. 31) en het Benin haplotype (Nr. 19).

Hoofdstuk 5. Dit hoofdstuk bestaat uit een algemene discussie van alle gerapporteerde onderzoeken en de bereikte conclusies. De mogelijke manieren waarop de waargenomen mutanten (β^s haplotypen Nr. 31, Nr. 19 en Nr. 20, en de deletie en nondeletie alpha-thal) in Kuwait en Nigeria zijn geïntroduceerd werden besproken. De belangrijkste conclusie is dat het mildere verloop van de ziekte in Kuwait is gerelateerd aan het β^s haplotype met een daaraan gekoppelde verhoging van het HbF en de hogere frequentie van het tegelijk voorkomende alpha-thal.

De gegevens die gepresenteerd worden in dit proefschrift laten zien dat sikkelcelanemie in Kuwait een mildere ziekte is dan in Nigeria. De frequentie van splenomegalie in stabiele fase van de ziekte is gelijk in beide groepen maar wanneer vergroting optreedt dan is deze veel sterker in Nigeriaanse patiënten. Het dominante β^s haplotype in Nigeria is het Benin haplotype (Nr. 19), terwijl in Kuwait dat het Saudi Arabisch/Indische (Nr. 31) is. Terwijl de frequentie van de alpha-thal-2(3,7 kb deletie) gelijk is bij beide groepen (ongeveer 25%) wordt bij de patiënten uit Kuwait bovendien in ongeveer 13% nondeletie alpha-thal gevonden. Bij de laatste groep is alpha-thal geassocieerd met normale miltfunctie en afwezigheid van ernstige herhaalde infecties en galstenen. Hetzelfde kon niet worden gevonden bij de Nigerianen. het lijkt er dan ook op dat het mildere verloop van de ziekte in Kuwait een gevolg is van het Saudi Arabische/Indische haplotype en de hogere frequentie van alpha-thal determinanten.

CURRICULUM VITAE

Name: Adekunle D. Adekile

Date and Place of Birth: 27th December 1949; Ibadan, Nigeria

Sex: Male

Citizenship: Nigerian

Education: University of Ibadan, Nigeria, MBBS - 1973

Postgraduate Qualifications: Fellow, Nigerian Postgraduate Medical College - 1981
Fellow, West African College of Physicians - 1982

Postdoctoral Fellowships: Clinical Fellow (Pediatric Hematology), Howard University Hospital, Washington, DC, USA; 1980 - 1981
Research Associate (Tissue Culture), Department of Zoology, Howard University; 1986
International Research Fellow, Department of Biochemistry and Molecular Biology, Medical College of Georgia, Augusta, GA, USA; 1990 - 1993.

Present Position: Associate Professor, Department of Paediatrics
Faculty of Medicine, Kuwait University
P.O. Box 24923, Safat 13110
Kuwait

List of Publications on Hemoglobinopathies

1. Jenkins, M.E., Reindorf, C.A., and Adekile, A.D.: The lungs in sickle cell disease. In: *Disorders of the Respiratory Tract in Children*, edited by E. Kendig and V. Chernick, page 84, W.B. Saunders Company, Philadelphia, 1983.
2. Adekile, A.D. and Makanjuola, D.I.: Realtime gallbladder ultrasonography in children with sickle cell anemia. *Nig. J. Paediatr.*, 10:35, 1983.
3. Adekile, A.D., Ogunye, O.O. and Olusi, S.O.: Alpha-1-antitrypsin phenotypes in sickle cell anaemia. *East Afr. Med. J.*, 61:136, 1985.
4. Adekile, A.D., Oyebola, D.D.O., and Olusi, S.O.: Oral glucose tolerance test in children with sickle cell anaemia. *East Afr. Med. J.*, 62:213, 1985.

5. Adekile, A.D., Haddy, T.B., Casto, O.L., and Scott, R.B.: Correlation of serum ferritin with other tests of iron status in sickle cell disease. *Am. J. Pediatr. Hematol./Oncol.*, 7:295, 1985.
6. Makanjola, D.I. and Adekile, A.D.: Realtime ultrasonography of the liver and spleen in paediatric HbSS patients. *Nig. Quart. J. Hosp. Med.*, 3:36, 1985.
7. Adekile, A.D.: Experience with cholelithiasis in Nigerian sickle cell patients. *Am. J. Pediatr. Hematol./Oncol.*, 7:261, 1985.
8. Adekile, A.D. and Thurnham, D.: Dependence of pyridoxine metabolism on riboflavin status in sickle cell patients. *Am. J. Clin. Nutr.*, 46:86, 1987.
9. Odetoyinbo, O. and Adekile, A.D.: Sensorineural hearing loss in children with sickle cell anaemia. *Ann. Oto-Rhino-Laryngol.*, 96:258, 1987.
10. Osifo, B.O.A., Lukambi, F.A., and Adekile, A.D.: Plasma cortisol in sickle cell disease. *Acta Haematol.*, 79:44, 1988.
11. Adekile, A.D., Adeodu, O.O., Jefe, A.O., and Odesanmi, W.O.: Persistent splenomegaly in sickle cell disease: Relationship to malaria. *Ann. Trop. Paediatr.*, 8:103, 1988.
12. Walker-Jones, D.G., Reindorf, C.A., Massac, E., Jr., Adekile, A.D., Hall, M.G., and Nunez, A.I.: Generation of differentiating human epidermal cells on basement membrane matrigel: Potential for use as autografts in recurrent ulcerative lesions. *Mat. Res. Soc. Symp. Proc.*, 110:393, 1989.
13. Walker-Jones, D.G., Reindorf, C.A., Massac, E., Jr., Adekile, A.D., Harmon, G.L., and Stanley-Ambrose, A.: Model for phase III autografts of epidermal cells cul-tured on a collagen-proteolycan matrix. *J. Natl. Med. Assoc.*, 81:1071, 1989.
14. Adekile, A.D.: Arterial oxygen tension, HbF and red cell 2,3 diphosphoglycerate in sickle cell anaemia patients with digital clubbing. *Ann. Trop. Paediatr.*, 9:165, 1989.
15. Adelekan, D.A., Adekile, A.D., and Thurnham, D.: Antioxidant capacity of the serum of sickle cell anaemia patients. *Eur. J. Clin. Nutr.*, 43:609, 1989.
16. Reindorf, C.A., Walker-Jones, D.G., Adekile, A.D., Oluwole, S.F., and Lawal, O.A.: Rapid healing of sickle cell leg ulcers treated with collagen dressing. *J. Natl. Med. Assoc.*, 81:866, 1989.
17. Adeodu, O.O., Adekile, A.D., Jeje, O.A., and Adedeji, F.A.: Serum immunoglobulins A and M in Nigerian sickle cell patients. *East Afr. Med. J.*, 66:631, 1989.
18. Olanrewaju, D.M. and Adekile, A.D.: Anthropometric measurements of sickle cell anemia patients in Ile-Ife, Nigeria. *Nig. Med. Pract.*, 10:35, 1989.
19. Adekile, A.D., Okonofua, F.e., and Durosinmi, M.E.: Pattern of menstruation in sickle cell anaemia patients. *Nig. Med. J.*, 20:26, 1990.
20. Adekile, A.D., Adeodu, O.O., Fadulu, S.O., Weinheimer, A., and Saduja, D.: Preliminary clinical trial of NX-06999 in the management of sickle cell anaemia. *Nig. Med. J.*, 20:44, 1990.
21. Adeodu, O.O. and Adekile, A.D.: Clinical and laboratory features associated with persistent splenomegaly in sickle cell anaemia patients. *Acta Haematol. Scand.*, 79:687, 1990.
22. Ogunleye, I.O., Adekile, A.D., and Fakunle, J.B.: Serum alkaline phosphatase in sickle cell anaemia. *Clin. Chim. Acta*, 190:291, 1990.

23. Adekile, A.D.: Interaction of malaria and Hb S in Nigeria. *Sphere*, 14(1):1, 1990.
24. Adekile, A.D. and Jegede, A.O.: Juvenile-onset diabetes mellitus in a sickle cell anaemia patient. *East Afr. Med. J.*, 67:591, 1990.
25. Oluwole, A.F., Asubiojo, D.I., Adekile, A.D., Filby, R.H., Bragg, A., and Grimm, C.I.: Trace elements in the hair of some sickle cell anaemia patients and controls. *Biol. Trace Elements Res.*, 26/27:479, 1990.
26. Adekile, A.D. and Akinseye-Akintujoye, O.: Serum amylase levels in sickle cell patients. *J. Natl. Med. Assoc.*, 83:459, 1991.
27. Adekile, A.D., Adeodu, O.O., Dairo, B.O., Johnson, W., and Reindorf, C.A.: Pitted red cell counts in Nigerian HbSS children: Relationship to age and splenic size. *J. Natl. Med. Assoc.*, 83:329, 1991.
28. Li, H-W., Codrington, J.F., Schiliro, G., Wadsworth, L.D., Beris, Ph., Adekile, A., and Huisman, T.H.J.: The usefulness of sequence analysis of amplified DNA for the identification of δ chain variants. *Hemoglobin*, 15:77, 1991.
29. Adekile, A.D.: Anthropology of the β^S gene-flow from West Africa to North Africa, the Mediterranean, and Southern Europe. *Hemoglobin*, 16:105, 1992.
30. Oner, C., Dimovski, A.J., Olivieri, N.F., Schiliro, G., Codrington, J.F., Fattoum, S., Adekile, A.D., Oner, R., Yuregir, G.T., Altay, Ç., Gurgey, A., Gupta, R.B., Jogessar, V.B., Kitundu, M.N., Loukopoulos, D., Tamagnini, G.P., Ribeiro, M.L.S., Kutlar, F., Gu, L-G., Lanclos, K.D., and Huisman, T.H.J.: β^S Haplotypes in various world populations. *Hum. Genet.*, 89:99, 1992.
31. Ramachandran, M., Gu, L-H., Wilson, J.B., Kitundu, M.N., Adekile, A.D., Liu, J-C., McKie, K.M., and Huisman, T.H.J.: Hb Muscat or $\alpha_2\beta_232(B14)Leu \rightarrow Val$ observed in an Arabian family in association with Hb S. *Hemoglobin*, 16:259, 1992.
32. Adekile, A.D., Kitundu, M.N., Gu, L-H., Adeodu, O.O., Lanclos, K.D., and Huisman, T.H.J.: Haplotypes in SS patients from Nigeria; characterization of one atypical β^S haplotype #19 (Benin) associated with elevated Hb F and high G γ levels. *Ann. Hematol.*, 65:41, 1992.
33. Adekile, A.D., Liu, J-C., Sulzer, A.J., and Huisman, T.H.J.: Frequency of the α -thalassemia-2 gene among SS patients from Nigeria and its influence on malaria antibody tiers. *Hemoglobin*, 17:73, 1993.
34. Adekile, A.D., McKie, K.M., Adeodu, O.O., Sulzer, A.J., McKie, V.C., Kutlar, F., Ramachandran, M., Kaine, W., Akenzua, G.I., Akolo, A.A., Asindi, A.A., Obinyan, E.A., Ogala, W.N., Ibrahim, M., and Huisman, T.H.J.: The spleen in sickle cell anemia: Comparative studies of Nigerian and U.S. patients. *Am. J. Hematol.*, 42:316, 1993.
35. Adekile, A.D. and Huisman, T.H.J.: Hb F in sickle cell anemia. *Experientia*, 49: 16, 1993.
36. Adekile, A.D., Dimovski, A.J., Oner, C., Lanclos, K.D., and Huisman, T.H.J.: Haplotype-specific sequence variations in the locus control region (5' hypersensitive sites 2, 3, 4) of β^S chromosomes. *Hemoglobin*, 17:475, 1993.
37. Adekile, A.D. and Huisman, T.H.J.: The level of fetal hemoglobin in children with sickle cell anemia: The influence of gender, haplotype and α -thalassemia-2 trait. *Acta Haematol.*, 90:34, 1993.
38. Dimovski, A.J., Adekile, A.D., Divoky, V., Baysal, E., and Huisman, T.H.J.: Polymorphic pattern of the (AT) χ (T) γ motif at -530 5' to the β globin gene in

- over 40 patients homozygous for various β -thalassemia mutations. *Am. J. Hematol.*, 45:51, 1994.
39. Dimovski, A.J., Divoky, V., Adekile, A.D., Baysal, E., Wilson, J.B., Prior, J.F., Raven, J.L., and Huisman, T.H.J.: A novel deletion of ~ 27 including the β -globin gene and the locus control region 3'HS-1 regulatory sequence: β^0 -Thalassemia or hereditary persistence of fetal hemoglobin? *Blood*, 83:822, 1994.
 40. Efremov, G.G., Dimovski, A.J., Baysal, E., Ye, Z., Adekile, A.D., Ribeiro, M.L.S., Schiliro, G., Altay, Ç., Gurgey, A., Efremov, G.D., and Huisman, T.H.J.: Possible factors influencing the haemoglobin and foetal haemoglobin levels in patients with β -thalassaemia due to a homozygosity for the IVS-I-6 (T $\rightarrow$ C) mutation. *Br. J. Haematol.*, 86:824, 1994.
 41. Dimovski, A.J., Adekile, A.D., and Huisman, T.H.J.: The *in vivo* expression of the globin genes of the β cistron in γ -, δ -, and $\delta\beta$ -thalassemia heterozygotes. *Experientia*, 50:167, 1994.
 42. Adekile, A.D. and Huisman, T.H.J.: Sick cell disease and other hemoglobinopathies. In: *Fundamental of Medical Cell Biology*, edited by E.E. Bittar, JAI Press, Inc., Greenwich, CT, in press.
 43. Boletini, E., Svobodova, M., Divoky, V., Baysal, E., Çürük, M.A., Dimovski, A.J., Liang, R., Adekile, A.D., and Huisman, T.H.J.: Sick cell anemia, sickle cell β -thalassemia, and thalassemia major in Albania: Characterization of mutations. *Hum. Genet.*, 93:182, 1994.
 44. Adekile, A.D., Yuregir, T.Z., Walker, E.L.D., III, Gu, L-H., Baysal, E., and Huisman, T.H.J.: Factors associated with hypochromia and microcytosis among high school students in the Southeastern USA. *South. Med. J.*, 87: 1132, 1994.
 45. Adekile, A.D.: Factors associated with the distribution and severity of sickle cell disease in Africa. *Sphere*, 17:1, 1994.
 46. Adekile, A.D., Gu, L-H., Baysal, E., Haider, M.Z., Al-Fuzae, L., Aboobacker, K.C., Al-Rashied, A., and Huisman, T.H.J.: Molecular characterization of α -thalassemia determinants, β -thalassemia alleles, and β^S haplotypes among Kuwaiti Arabs. *Acta Haematol.*, 92:176, 1994.
 47. Baronciani, L., Magalhães, I.Q., Mahoney, D.H., Westwood, B., Adekile, A.D., Lappin, T., and Beutler, E.: Study of the molecular defects in pyruvate kinase deficient patients affected by nonspherocytic hemolytic anemia. *Blood Cells Molec. Dis.*, 21:49, 1995.
 48. Adekile, A.D., Kazanetz, E.G., Leonova, J.Ye., Marouf, R., Khmis, A., and Huisman, T.H.J.: Co-inheritance of Hb D-Punjab (codon 121; GAA $\rightarrow$ CAA) and β^0 -thalassemia (IVS-II-1; G $\rightarrow$ A). *Am. J. Pediatr. Hematol./Oncol.*, in press.
 49. Leonova, J.Ye., Kazanetz, E.G., Smetanina, N.S., Adekile, A.d., Efremov, G.D., and Huisman, T.H.J.: Variability in the fetal hemoglobin level of the normal adult. *Am. J. Hematol.*, in press.
 50. Adekile, A.D., Tuli, M., Haider, M.Z., Al-Zaabi, K., Mohammadi, S., and Ozunwanne, A.: Influence of α -thalassemia trait on spleen function in sickle cell anemia patients with high Hb F. *Am. J. Hematol.*, in press.
 51. Adekile, A.D. and Haider, M.Z.: Morbidity, β^S haplotypes, and α -globin gene patterns among SS patients in Kuwait. *Acta Haematol.*, in press.