MEDICAL GRAND ROUNDS

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The Thalassemia Syndromes: Recent Advances in Molecular Mechanisms and Management

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I. INTRODUCTION

The thalassemias are a group of hereditary disorders of hemoglobin synthesis. Several population groups have a high incidence of these defects and therefore they produce a major health problem in several areas of the world, including those areas of the United States where there is a high concentration of persons of such ancestries. The clinical manifestations of these disorders were reviewed at these rounds approximately ten years ago. The message at that time was that a major amount of descriptive data was available but that no significant inroads had been made into the clinical management of those individuals homozygous for these disorders.

In the past decade significant advances have been made in the therapeutic approaches to these disorders. In addition, innovative technology has allowed detailed investigation of the molecular pathology at the gene level. It is the intent of this exercise to review the therapeutic advances, and, in addition, to summarize the molecular genetics of these disorders emphasizing how the basic technology is being applied towards developing alternate stratagems of management of these diseases.

II. THE BASIC PROBLEM

All of the thalassemias have in common the feature of a genetically determined reduction in the synthesis of the normal polypeptide chains of hemoglobin. The hemoglobin molecule is composed of four polypeptide chains, each possessing a single heme group. All known functional hemoglobins after birth are composed of a pair of alpha polypeptide chains and a pair of non-alpha chains; the latter, because of differences in amino acid sequences, determining the type of hemoglobin. At least six normal hemoglobins are presently recognized beginning in early fetal life. Only three are of significance to the present discussion: a) hemoglobin F, the predominant hemoglobin during fetal life, and during the first several months of postnatal life; b) hemoglobin A, the predominant hemoglobin from approximately one year of life and throughout adulthood; and c) hemoglobin A_2, a minor hemoglobin which reaches its maximum quantity at approximately the same time as hemoglobin A. From the relative amounts of these hemoglobins, it is clear that functional oxygen carrying capacity requires the production of adequate amounts of alpha chains during both fetal and postnatal life and equivalent amounts of γ or β chains during fetal and postnatal life respectively. The structural genes for the polypeptide chains behave

Table 1

Normal Adult Hemoglobin

Type	Structure	% of Total Hemoglobin
A	$\alpha_2\beta_2$	>95
A_2	$\alpha_2\delta_2$	2.5
F	$\alpha_2\gamma_2$	< 2

as Mendelian co-dominants. Therefore, the transcription of messenger RNA (m RNA) by allelic structural genes is approximately equivalent. In addition, the intracellular translation of alpha and non-alpha polypeptide chains is essentially balanced in normal erythroid elements. These two features of hemoglobin polypeptide chain synthesis are fundamental to normal erythroid cell function and survival. The thalassemia syndromes are a direct consequence of alterations of these elements.

Since most of postnatal life requires adequate amounts of hemoglobin A, the thalassemia disorders which are clinically significant are those in which either alpha chain synthesis is impaired (α -thalassemias) or beta chain synthesis is effected (β-thalassemias). The clinical features of the heterozygous, homozygous, multiple heterozygous and heterozygous conditions for thalassemias and abnormal hemoglobins were reviewed at these G.R. in 1970 and are thoroughly discussed in the monograph by Weatherall and Clegg (1).

The clinical manifestations are a consequence of two pathogenetic alterations of the previously mentioned elements of hemoglobin synthesis. A reduction in globin chain synthesis produces cells deficient in total hemoglobin concentration, ie. a microcytic/hypochromic state. The greater severity of these disorders as compared to other types of hemoglobin synthetic abnormalities is a consequence of the selective decrease of one of the two major polypeptide chain pools ie. imbalanced globin chain synthesis (2). Excess quantities of alpha or beta chains form intracellular tetramers of α 4 or β4 (3). Besides being nonfunctional, these structures are highly insoluble or unstable resulting in their precipitation with the cell (Heinz bodies), attachment of the inclusion bodies to the cell membrane and subsequent removal of a part or all of the inclusion body containing cell (4). The consequence of this series of events is either pre-mature intra-medullary destruction of developing erythroblasts (ineffective erythropoiesis) or early destruction of mature circulating erythrocytes (hemolysis) (5). Thus, imbalanced globin chain synthesis is the key to the magnitude of the disorder in a given patient. Clinically, the disorders are described in terms of the severity of the anemia (correlated with the degree of globin chain imbalance). If the decrease in hemoglobin content is the major abnormality this is termed thalassemia minor. Where moderately severe ineffective erythropoiesis and hemolysis are present, the disorder is termed thalassemia intermedia (heterozygotes for two types of thalassemia, rarely heterozygotes and at times homozygotes for some forms) and where these features are very severe the disorder is called thalassemia major (homozygotes for most forms of thalassemia).

Erythrokinetic studies reveal the magnitude of these pathogenetic effects on normal effective erythropoiesis and red cell survival (Table 2) (4, 5).

Table 2 Representative Erythrokinetic Values in β Thalassemia Major

Parameter	Normal	β Thalassemia
Mean RBC life span (days)	120	12
Size of erythron	1	10-30
Effective RBC production	1	1
Ratio effective/total production	0.85-1.00	0.05-0.15

III. CONSEQUENCES OF THE PATHOGENETIC FEATURES

The 10-30 fold expansion of the marrow cavity results in a number of the phenotypic features of young children with thalassemia major. These include the widening of the skull and facial bones producing the characteristic facies, orthodontic abnormalities and skeletal fractures (6-8). The anemia is clearly responsible for the problems with exercise tolerance, cardiomegaly and hepatosplenomegaly (due to extramedullary erythropoiesis) in early years and appears to be responsible, in part, for the early growth retardation (8, 11). The iron overload state secondary to enhanced absorption and transfusional requirements is responsible for the remaining manifestations, especially those producing the greatest morbidity and ultimately, mortality. Endocrine abnormalities, in most instances, are subclinical in their manifestations, but can be demonstrated by appropriate functional studies. However, overt diabetes mellitus and failure of appropriate gonadotropin production are clinically demonstrable, the latter expressing itself as a failure of normal puberty. Lack of the expected growth spurt, menses and development of secondary sexual characteristics are appreciated in most patients. Data from Nienhuis et. al. and Kletsky et. al. support that the pubertal failure is due to insufficiency of the pituitary release of LH and FSH (12, 13). Subclinical evidence for hypofunction of the thyroid and adrenal glands has also been obtained in older patients (12, 13). Cirrhosis is the rule in older patients. In patients who die from the consequences of iron overload, all demonstrate cirrhosis at post-mortem examination, although overt liver failure is rarely the cause of death (14). The cardiac effects of iron overload are responsible for the greatest morbidity and are the single largest cause of mortality in thalassemia major (14, 15). Virtually all patients with transfusion dependent thalassemia major die of their disease, usually by 24 years of age. As transfusional support has been readily available in the past few decades, iron overload has become a major mode of demise. Causes of death in 52 cases reviewed by Modell since 1959 are listed in Table 3 (14).

Table 3
Ref 14

CAUSE OF DEATH IN THALASSEMIA MAJOR

Total deaths		52
Hemochromatosis	21	
Other	31	
Anemia in infancy		
Infection		
Thrombocytopenia		

All 21 cases who died of iron overload succumbed to resistant cardiac failure and arrhythmias. The development of cardiac failure was almost invariably preceded by a predictable pattern of events as listed in Table 4. These deaths occurred from ages 14-24. In several patients, other overt endocrine disorders (eg. diabetes mellitus) and pericarditis also preceded the development of overt cardiac decompensation. The pattern of cardiac abnormalities

Table 4 Ref 14 — Clinical Manifestations of Patients Dying of Hemochromatosis in Thalassemia Major

Failure of Pubertal Growth

Absence of Sexual Development

Massive Hepatomegaly

Heart Failure

in thalassemia major has been reviewed by Engle, et. al. (15) (Table 5). At post mortem in both these series, the pathologic findings were essentially those described by Buja and Roberts in patients succumbing to cardiac iron overload (16).

Table 5 Ref 15 — Cardiac Abnormalities in 46 Patients Over Age 8 With Transfusion Dependent Thalassemia Major

Manifestation	Percent Effected
Cardiomegaly	85
Pericarditis	41
Heart Failure	52
Supraventricular Arrhythmias	37
VPB	26
Ventricular Tachycardia	7
Complete Heart Block	4
Cardiac Death	50

IV. THERAPEUTIC APPROACHES TO THALASSEMIA MAJOR

A. Transfusion Therapy

Since most of the early clinical manifestations of this disease can be traced to the consequences of the marked bony expansion secondary to the massive ineffective erythropoiesis and the anemia per se, attempts at more vigorous transfusion therapy became a consideration for management. Classically, patients were transfused only when major symptomatic anemia existed (at the range of 5-6 gm/dl hemoglobin). In 1964, Wolman published a retrospective analysis of young patients treated with transfusions in whom the pediatricians utilized different pre-transfusion hemoglobin criteria (8). Table 6 summarizes his observations. The resulting enthusiasm led to the employment of this practice at most centers caring for these children, utilizing a rather wide

range of criteria for pre-transfusion red cell values. With few exceptions the effect on growth, bone abnormalities, hepatosplenomegaly, cardiomegaly and exercise tolerance have been dramatic. The children are able to live essentially normal lives and participate in athletic endeavors with only minor skeletal disfigurement during their early years (9-10, 14, 17, 20). Unfortunately, from about the age of 8-11, there is a rather abrupt failure of growth and lack of development of the expected secondary sexual features (11, 14, 17). This clearly indicates the clinical development of early manifestations of iron overload. Measurements of cardiac wall thickness and myocardial function are compatible with this thesis (11, 84).

Table 6
Ref 8

Effects of Hypertransfusion Therapy on Signs and Symptoms in Thalassemia Major

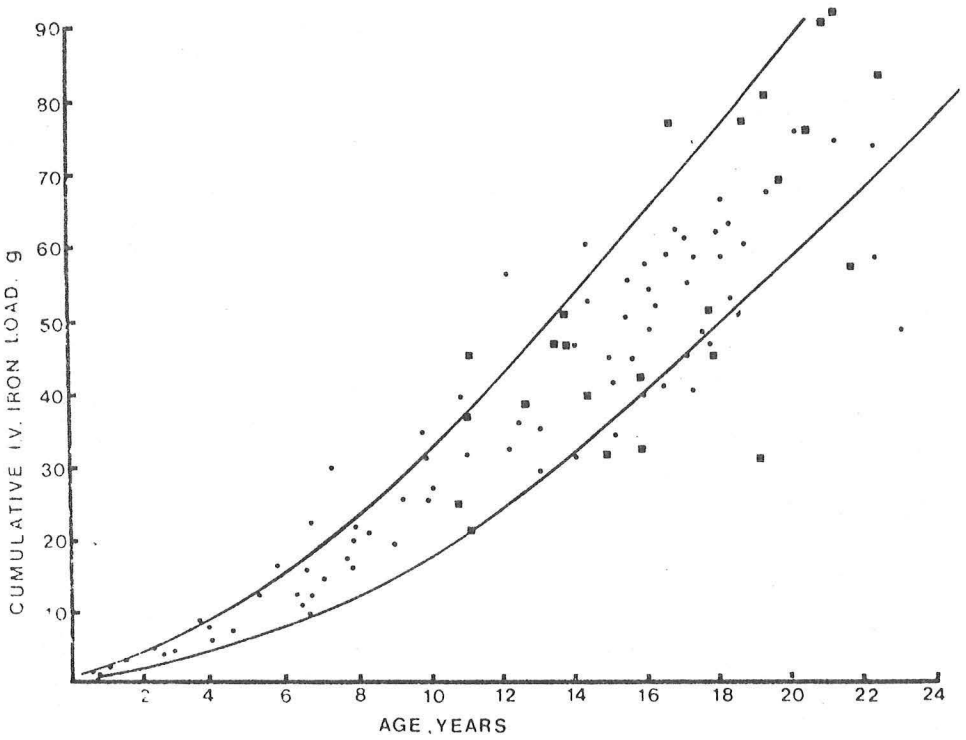
	Average Pre-Transfusion Hgb Conc (gm/dl)		
	4.0-5.9	6.0-7.9	8.0-9.9
Average Age (years)	8.0	8.0	7.4
Height (mean percentile)	4.9	7.6	14.1
Weight (mean percentile)	26.4	23.1	23.7
Skeletal age (mean percentile)	24.7	20.7	18.3
Liver (cm below costal margin)	10.2	9.6	4.7
Spleen (cm below costal margin)	14.3	8.2	5.3
Skull thickening (cm)	14.9	13.9	10.7
Fractures (% pts)	25	15	0
Orthodontic problems	92	54	30
Cardiomegaly (% pts)	80	38	11

B. Management of Iron Overload

The problems of the early years for patients with thalassemia major appear relatively well ameliorated by hypertransfusion regimens. Nevertheless, chronic iron overload is predictable and, with it, the ultimate morbidity and mortality associated with this problem. It is of interest that the rate of development of iron excess is not overly exaggerated by this approach, probably for two reasons. First, iron absorption is significantly suppressed because of the suppression of a major portion of erythropoiesis (10, 14, 18, 19) and secondly, the amount of blood necessary to maintain these levels has been less than initially predicted (10, 20).

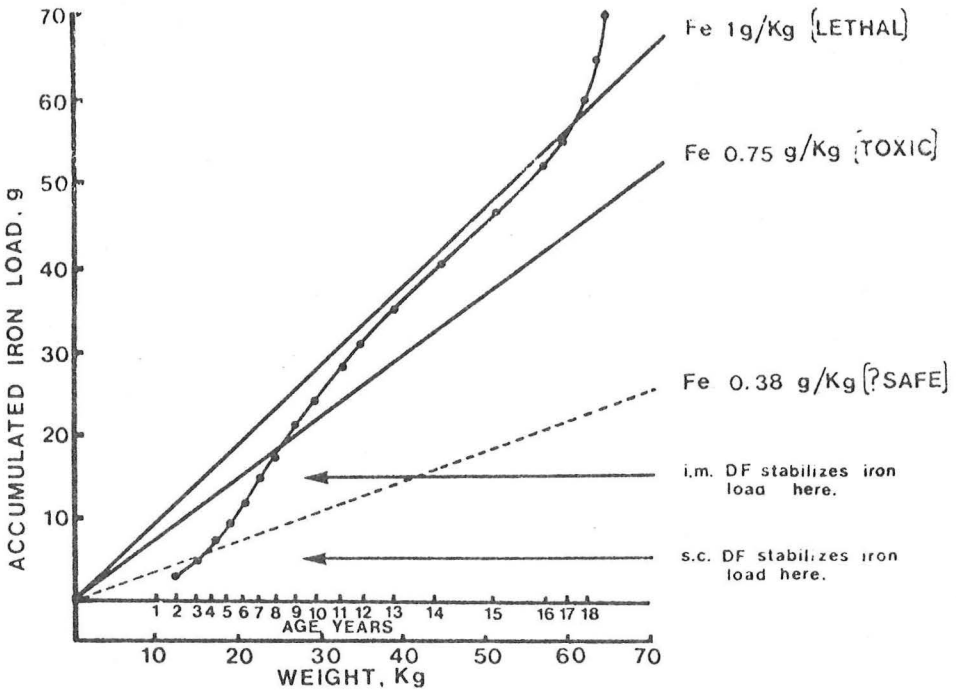
The most efficient means of removing iron from patients is by phlebotomy. Obviously, this is not possible in this type of disorder. The alternative approach was to investigate the utility of administering iron chelating agents on a chronic basis. Deferoxamine (DFO), a complex hydroxylamine produced by

Streptomyces pilosus in rat culture, with a molecular weight of 657 daltons, was chosen for this purpose because it is the most efficient iron chelator with the least toxicity that is presently available (21). It binds approximately 85 mgm of iron per gram of the methane sulfonate salt. Solubility is limited to approximately one gram in 3.5 ml volume and must be administered parenterally. These solubility factors, therefore, limit the quantity that can be administered by intramuscular injection. The drug presumably enters cells and binds iron from the so-called "labile pool" predominantly in R.E. cells (22). Since there is a relatively good correlation between DFO induced iron excretion and total body iron load, the size of this pool appears to be proportional to total intracellular non-hemoglobin iron (23). Approximately 1/3 of the iron is excreted in the stool and 2/3 in the urine (24). The earliest studies in thalassemia major were carried out using the intramuscular route and the largest experience has been that of Modell (14, 25). The potential effectiveness of this approach must be related to the daily rate of iron accumulation (Figure 1), the total body iron load which produces toxic manifestations (Figure 2 and Table 7) and the amount of iron excreted by chelation at a given level of iron excess (Figure 3).



The cumulative iron load received intravenously as blood in high-transfused patients with thalassemia major. The upper line represents the rate of loading of a child growing on the 97th centile; the lower line is the rate of loading of one growing on the 3rd centile. By the age of 10 the average patient has received 25 grams of iron intravenously, and by the age of 17, about 60 grams. The curve shown does not allow for pubertal growth since this has hitherto been reduced or absent in most patients with thalassemia. Each point represents the accumulated iron load of an individual patient. Patients now dead are marked by squares. Points considerably above the upper line indicate long-standing hypersplenism; points below the lower line indicate a very low transfusion scheme.

Fig. 1
Ref. 14



Cumulative iron load and iron toxicity in thalassemia major. An iron load of more than one gram per kilogram may lead to death, and an iron load of more than 0.75 grams per kilogram may produce toxic symptoms. The "lethal" level is graphed in relation to body-weight and age (upper solid line) while the "toxic level" is the lower solid line. It may reasonably be supposed that half the load known to produce toxic symptoms, i.e. less than 0.38 grams per kilogram, represents a safe level (lowest dotted line). The observed mean iron load in thalassemic patients from Fig. 3 is superimposed on these three lines. Until the age of three the body iron load in thalassemia remains within the "safe" range; it then crosses this boundary and enters the range known to produce toxic symptoms by about 9 years of age. This corresponds well with the observed growth retardation starting at 11 years of age in these patients. Due to the dilutional effects of growth, the iron load remains between the toxic and lethal ranges for a number of years. If the patient passes through puberty, this period of time is substantially increased. (A normal pubertal growth spurt is allowed for in this chart). As soon as growth ceases the accumulated iron load rises steadily into the known lethal range. A similar graph could be made for Diamond-Blackfan anemia and would show the iron load entering the known lethal range by 11-to-12 years of age.

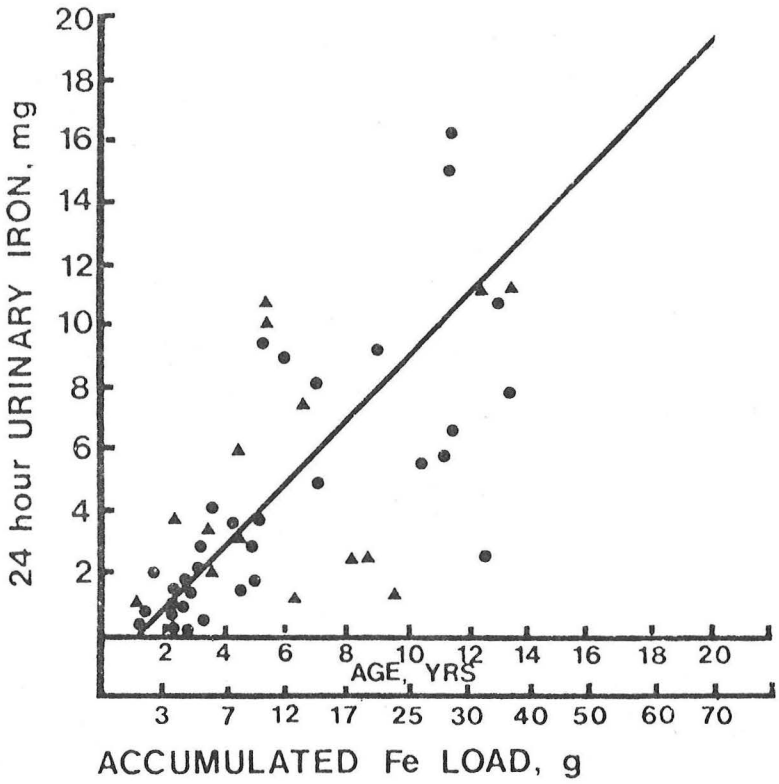
Fig. 2
Ref. 14

The body iron load which produces toxic effects is relative to the size of the patient and can be expressed in terms of body weight. Thus Modell and her colleagues found that severe growth regardation and failure of sexual development (the earliest clinical expression of toxicity) were seen in those patients whose accumulated iron load was in the range of 0.75-1.0 g/Kg (mean 0.83). This averaged approximately 27.5 gm, at about the age of 11, where the average weight was 33 kg. Iron load associated with death was assessed by calculating transfusional quantities administered as well as iron content

Table 7 Iron Content of Body and Tissues in Patients with Thalassemia Major

	Body Iron		Tissue Iron	
	Total (grams)	Concentration (gm/Kg)	Total (grams)	Concentration % dry wt
Toxic Effects				
Growth	25-30	0.75-1.0	-	-
Liver*	20	-	?	2-4
Heart	-	-	?	?
Fatal	40-80	1.0-1.6	-	-
Liver	-	-	40-75	3.5-9.0
Heart	-	-	0.8-1.3	0.6-1.3

* Gross hepatic enlargement



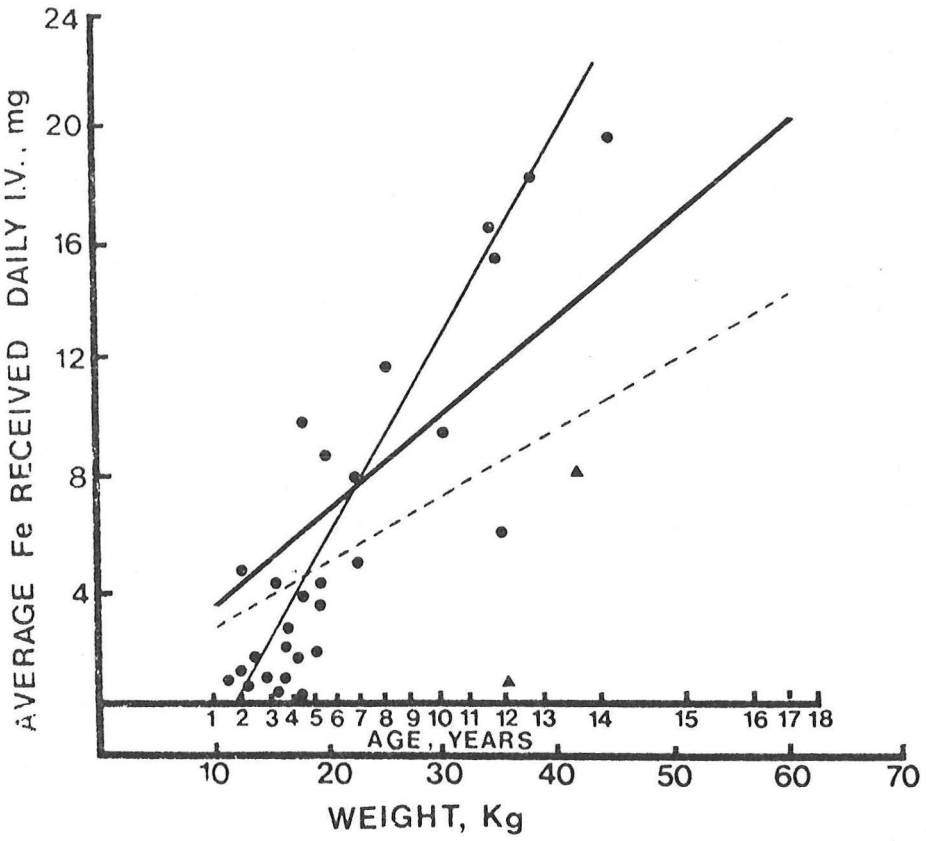
Observed urinary iron excretion after 500 mg deferoxamine given intramuscularly related to age and accumulated iron load in thalassemia. The circles are the author's observations, the triangles are the original observations of Sephton Smith [75]. These observations were made in patients who had not been receiving ascorbic acid.

Fig.3
Ref. 14

of tissues at post mortem. The majority of patients succumbing to the toxic effects of iron had received over 40 gm via transfusion. In the few cases where less had been received, tissue iron content indicated that there had been significant amounts acquired via G.I. absorption. Iron levels in these patients are summarized in table 7. It is clear that total body iron loads exceeding 1 gm/Kg are associated with toxic deaths. It would therefore seem reasonable that if loads of 0.75-1.0 gm/Kg are associated with clinical manifestations and loads greater than this lead to death, an effective chelation regimen must provide for stabilization below these levels. It should be emphasized that these are minimum figures. Piomelli has noted increased ventricular wall thickness by echocardiography in children as young as age 3 who have never been anemic because of lifelong hypertransfusion therapy, presumably on the basis of myocardial iron accumulation (11). Also, it is not known whether chronic iron levels below those listed would eventually lead to functional abnormalities of liver, heart or endocrine organs.

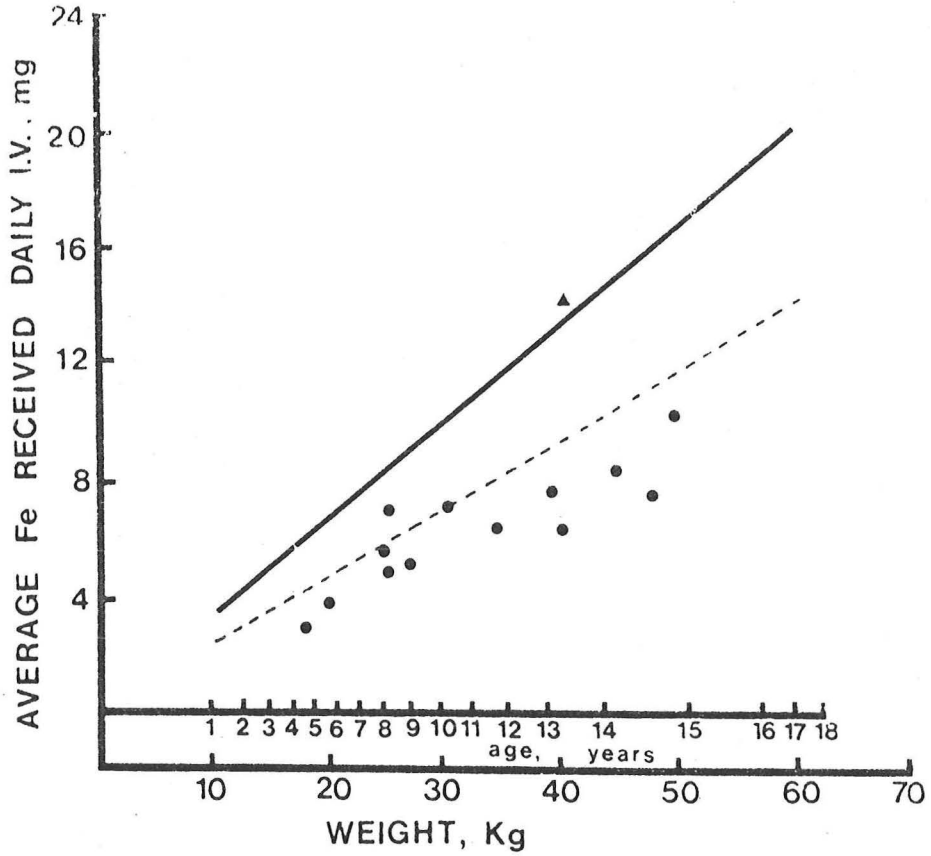
The results of the British studies are seen in figures 4 and 5. Urinary iron excretion is a function of the total accumulated iron load. Utilizing maximal tolerated doses of I.M. DFO (20 mgm/Kg/d), stable or negative iron balance is not reached until approximately the age of 5-7 years corresponding to a total load of 10-15 grms or 0.7 gm/Kg (Figure 4). This is only marginally below the observed toxic level. Maximal effectiveness of DFO can be achieved by using the highest tolerated doses and maintaining vitamin C repletion (see below). When doing this, they have demonstrated that near stable or only modestly positive iron balance can be achieved over a long period of time (Figure 5). Nevertheless, near toxic or eventually toxic levels of iron would subsequently be reached by most patients on chronic hypertransfusion therapy with the intramuscular route of administration of the chelating agent. As a consequence other investigators have pursued alternative routes of administration with the goal of further enhancing negative iron balance. Several workers have demonstrated that continuous I.V. DFO results in a greater excretion of iron than does I.M. for a comparable dose and total body iron load (11, 12, 26, 27). Clearly, daily intravenous administration is logistically not acceptable. Therefore, the utilization of the subcutaneous route was developed by Propper et. al. by employing a portable syringe pump which could be used by outpatients for a variable period of time on a daily basis (28). This route of administration is 80-100% as efficient as the I.V. route (11, 28, 29). Although there is a limitation to the total quantity of DFO which produces an increasing level of iron excretion (dose-response) (14), the time of infusion can be reduced so that the efficiency of a 12 hour infusion is approximately 80% and a six hour infusion 70% of a 24 hour continuous sub Q. regimen (30, 31). On an average, the net result is that heavily iron loaded patients can be put into negative iron balance, and more importantly, stable iron balance can be achieved in the children at approximately age 2-3 when the total body iron load reaches approximately 5 grams or 0.35 g/Kg body weight (Figure 2, 6). Long term studies indicate that the chelating efficiency of DFO persists with time (10).

Efficiency of DFO induced iron excretion can be further enhanced in some patients by the chronic administration of vitamin C (32, 33). The mechanism of action is unclear although it appears to increase the amount of iron



[redacted] Relationship between average daily iron intake (as blood), and body weight (and age) in thalassemia major. The theoretical blood requirement of thalassemic patients of different weights (expressed as mg of iron received intravenously per day) is graphed (upper heavy solid line). Since at least one-third of the iron excreted in response to deferoxamine appears in the stool, the amount of iron that must appear in the urine daily, in order to express iron balance, is shown as the lower dotted line. The observed urinary iron excretion in hitherto unchelated patients started on deferoxamine 500 mg/day is shown. Each circle represents the average daily iron excretion (measured over 3 days) of an individual patient. Younger patients are not brought into iron balance by this treatment, but older patients are brought into negative iron balance. The line representing observed iron excretion crosses the lines representing iron balance somewhere between 5 and 7 years of age. The accumulated iron load reached by this age (i.e. 10 to 15 grams) must, therefore, represent approximately the iron load that is held steady by long-term intramuscular deferoxamine therapy. Accumulation of iron beyond this point should be arrested. Reference to Fig. 2 shows that this iron load is around 0.7 grams per kilogram body weight, which is only marginally below the known toxic range.

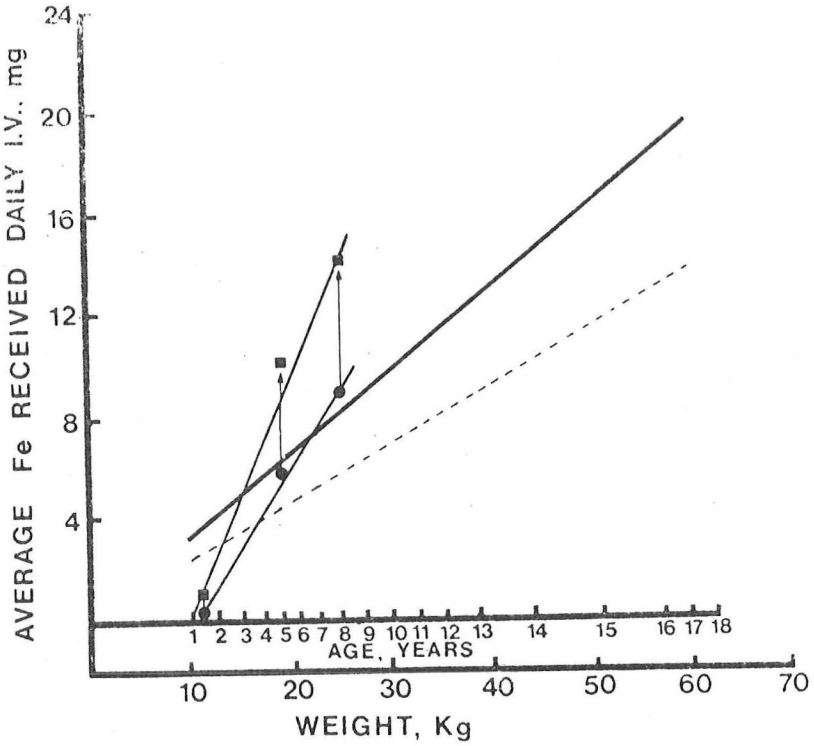
Fig. 4
Ref. 14



The urinary iron excretion of patients maintained for several years on intramuscular deferoxamine therapy (using the same convention as in Fig. [redacted]). Most patients are excreting an amount of iron in the urine approximately representing balance or slightly less. These patients probably excrete more than one-third of the chelated iron in their stool. The one patient (▲) who still excreted a large amount of iron in response to 500 mg of deferoxamine was not taking his injections regularly and had allowed his body iron to expand, as revealed by this high excretion.

Fig. 5
Ref. 14

available in the chelatable pool. The latter would suggest the potential for enhancement of iron toxicity as well, and case reports of acute cardiac decompensation when large doses of vitamin C have been given have appeared (12, 27). The enhancing effect appears to occur most notably in older and highly overloaded patients who tend to show evidence of ascorbic acid deficiency (perhaps as a consequence of the degree of iron excess) when WBC ascorbate levels are measured (32, 33). Low dose vitamin C supplementation during active chelation therapy has become a standard part of most regimens.



[redacted] Iron balance in young thalassemic patients on 500 mg of deferoxamine per day, given by intramuscular injection and subcutaneous infusion (Data from Pippard et al. [81]). The iron excretion when deferoxamine is given intramuscularly (●) corresponds to that shown in Figure [redacted]. When the same dose is given subcutaneously, (■) the response is greatly increased. With subcutaneous infusion, the line representing mean iron excretion intersects the line representing iron balance somewhere between 2 and 3 years of age, i.e. at a body iron load of less than 5 g. This represents approximately 0.35 g/kg body weight. An iron load of this order should be in the safe range.

Fig. 6
Ref. 14

The long term effects of iron chelation therapy on the consequences of iron overload are only assessable in a preliminary fashion at this time. Of great importance, toxicity to date appears only minimal. Some patients occasionally have local inflammatory reactions at the needle site but suppurative infections have been rare. Rarely, systemic allergic reactions have necessitated discontinuance of therapy. Long term animal studies have demonstrated the development of cataracts with DFO, but only one or two cases have been noted in humans and these have been reversible. Certainly, other effects may eventually be seen if the duration of therapy continues to increase (14). Since iron chelation therapy in a sizable number of patients has occurred for the longest period in Great Britain, most of the early data on survival and manifestations of iron toxicity have accrued from the I.M. studies of Modell (25) 24 hour ECG monitoring has been carried out on some of their patients. Results are summarized in table 8. The patients

Table 8 Results of Chronic Iron Chelation Therapy with Hypertransfusion for Thalassemia Major

Patients	# studied	# abnormality	%
Major arrhythmias on 24 h ECG monitor	28	7	25
Non-chelated	11	7	64
Chelated	17	0	0
More than 200 units blood	13	5	38
Non-chelated	6	5	83
Chelated	7	0	0
Deaths in children over 14 years	49	10	20
Non-chelated	30	9	30
Chelated	19	1	5
Deaths in children over 17 years	30	8	27
Non-chelated	20	8	40
Chelated	10	0	0

have been chelated for 7-10 years. Most were begun after significant iron overload would have occurred and therefore no data is yet available on the earliest manifestations of iron overload. Subsequently, a small randomized prospective study was begun in patients who were matched for age, sex, transfusion requirement and splenectomy status (29-31). There were ten patients in each group and results at the sixth year are summarized in Table 9. The trial was discontinued and the remaining control patients placed on chronic subcutaneous therapy (14).

Table 9 Results of Great Ormond Street Controlled Trial of I.M. DFO in Thalassemia Major

Parameter	Treated Group	Control Group
Number	10	10
Serum ferritin (ngm/ml)	6,100	10,500
Liver iron (% dry wt)	2-3	3.5-5.5
Liver fibrosis	Stable	Progressed
Deaths	1	6

It should be emphasized that these principles can be applied to transfusional iron overload that may occur in adults with acquired forms of anemia requiring long term high level transfusion therapy. In patients who have accumulated 14-70 grams of iron, the minimal net loss of iron by DFO administration will be in the range of 4-10 grams per year in those patients who have been chelated for 6-18 months (28).

C. Neocyte Transfusions

Standard transfused blood contains red cells with ages ranging from reticulocytes to near senescence with a mean life span of approximately 60 days. Red cells of differing age have different densities and can be separated into younger and older populations by differential sedimentation. With the development of the large batch cell separators, it is now possible to carry out such separation on a scale that can be applied to transfusion therapy. Propper et. al. have used this approach to obtain a fraction of red cells for transfusion that have a mean age of twelve days (mean life expectancy of 108±12 days) (34). Such an approach has several potential advantages: (1) transfusion rates will be only about one-half as frequent producing daily iron accumulation of 2-5 mgm; (2) the average hemoglobin levels would be even higher since this cohort of cells will reach its senescence almost simultaneously rather than on the usual daily fixed fractional decline; (3) using the cell separator, the near senescent red cells could be removed from the patient while administering the young cell cohort thus further reducing the iron accumulation; and (4) this reduced rate of iron accumulation could then be dealt with utilizing less efficient but also less expensive chelating agents at perhaps weekly intervals (14). The logistical problems are approachable, especially when considering the actual reduction in transfusion frequency and volume that would be achieved by such manipulation of transfused blood.

V. ALTERNATE STRATEGEMS

A. Molecular Genetics and Prenatal Diagnosis

During the past decade, major strides have been made in identifying the primary defects producing the thalassemia disorders (36). This has been the result of the development of innovative methodology in the fields of protein and nucleic acid chemistry. Not only have these techniques provided detailed analysis of the molecular alterations that result in thalassemia, they have uncovered a significant heterogeneity in the mechanisms which can produce the phenotypic expression termed thalassemia. An important byproduct of these basic laboratory studies has been the application of these techniques to certain aspects of the clinical management of these disorders, especially in the area of pre-natal diagnosis.

The problems of management of thalassemia major could be averted if pre-natal diagnosis could be achieved at a time early anough to allow therapeutic abortion to be safely performed. Utilizing techniques developed in the laboratories studying the molecular mechanisms of thalassemia, significant advances are being made in the practical application of this alternative. Although less exact and sensitive than for abnormal hemoglobin states, techniques are available for screening parents for the heterozygous thalassemia states that are relatively simple and inexpensive (35). High risk populations can be screened and where both potential parents are found to be heterozygous for thalassemia appropriate genetic counseling can be given. If a desire for children remains, certain severe forms of thalassemia can be detected sufficiently early *in utero* to permit the use of therapeutic abortion.

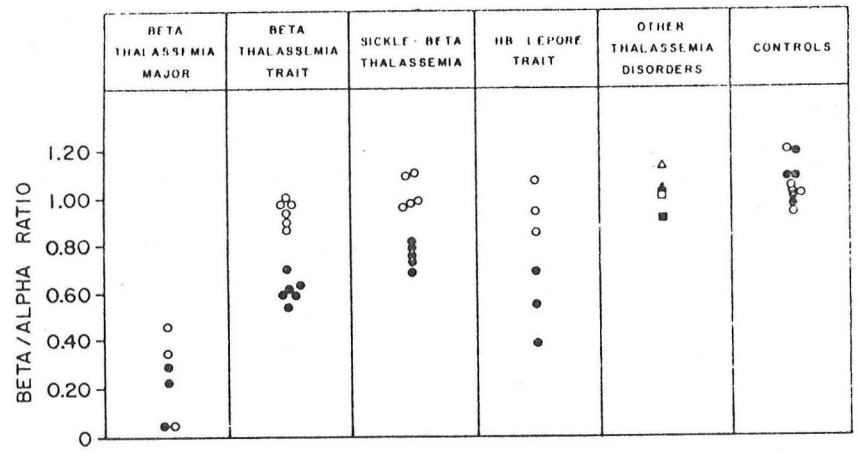
The major pathophysiology of the thalassemia disorders is a consequence of not only decreased globin chain synthesis, but more importantly, *imbalanced globin*

chain synthesis. This is a result of three primary features: a) the structural gene loci for the alpha globin chains and non-alpha globin chains are located on separate chromosones (α loci on chromosome 16 of the γ δ β complex on chromosome 11) (37, 38) (Figure 7); b) the various thalassemic mutations act only in cis position on and are allelic to one or the other complex (1) and c) the gene products of the unaffected loci can only be minimally modulated ie. decreased or increased globin chain synthesis by normal loci occurs only insignificantly (1). Incubation of normal bone marrow cells or peripheral blood reticulocytes with radiolabelled amino acids demonstrates a ratio of chain synthesis of alpha and non-alpha chains of near unity. In the beta thalassemias, the beta to alpha ratio is significantly reduced (39) (Figure 8). The severity of the disorder will be a consequence of the degree

Figure 7

The γ δ β Gene Complex

γG γA δ β



[redacted]. Globin synthesis ratios in controls and a variety of disorders with impaired hemoglobin synthesis. ●=peripheral blood; ○=bone marrow; ■=αβ-thalassemia (peripheral blood); □=αβ-thalassemia (bone marrow); ▲=Negro β-thalassemia trait (peripheral blood; △=Negro β-thalassemia trait (bone marrow).

Fig. 8
From Schwartz and Gill
Ann NYAS 232:33, 1974.

of reduction of synthesis of the affected β chains. Two general groups of beta thalassemias exist; those with a partial impairment of globin chain synthesis due to the primary genetic defect, called β+ thalassemias, and those with a total elimination of globin chain production ie. β^{o} thalassemia (1). The defects can be further delineated by isolating mRNA (messenger RNA) from normal or thalassemic bone marrow or reticulocytes and using that

as the primer in cell free systems for protein synthesis. Under these conditions the thalassemic defect is expressed as imbalanced chain synthesis which could be due either to decreased amounts of mRNA or non-functional mRNA (40-42). The next methodologic advance allowed an assessment of these alternatives. Globin mRNA can be isolated and enriched for α -mRNA or β-mRNA by a variety of means. This material can then be utilized as a template for the production of a single stranded complementary DNA for the appropriate globin chain (termed c-DNA) using the enzyme reverse transcriptase or cloning techniques. When mixed with normal or thalassemic mRNA, the specific c-DNA's will combine or anneal with the specific mRNA's at a rate that is dependent upon the amount of the specific mRNA's present and thus is a means of quantitating the amount of specific mRNA present in a given preparation. By employing this technique of hybridization with mRNA prepared from normal bone marrow cells or reticulocytes, the β to α mRNA ratios are approximately unity. At this point, the heterogeneity of the β thalassemias becomes even more apparent. In the β^+ thalassemias, there is usually identified a quantitative decrease in mRNA equivalent to the decrease in β globin chain synthesis. In most β^0 thalassemias, there is an absence of β-mRNA, but in some, there is a residual identifiable mRNA that hybridizes that is not functioning (43-46). The next step in the resolution was the development of techniques for gene mapping. Basically, this allows the identification of the structural gene loci for the various globin chains. The technology employs the technique of DNA hybridization and the application of a number of restriction endonucleases. These enzymes cleave DNA into oligonucleotides at base sequences (restriction sites) that are specific for each separate endonuclease. The resulting DNA fragments are then electrophoresed on agar-gel which allows their separation based on size. After blotting or directly, the DNA fragments are exposed to radiolabelled specific cDNA's and those fragments that contain the appropriate gene sequences are identified by auto-radiography (47). Complete or partial deletions of gene material will be identified by changes in the amount or size of the reacting DNA fragments. The relative number of globin genes can also be quantified using DNA hybridization in solution as was done for mRNA. A further advantage of this technique is that DNA from any cell type can be used. As would be predicted, the molecular heterogeneity of the thalassemias is further compounded by these techniques. In the β^+ thalassemias, a normal number of β gene loci are present indicating a decrease in transcription or nuclear processing of mRNA in the disorder (the capacity for translation of normal mRNA has been shown to be intact) (48). A partial summary of some of the defects seen in the β thalassemias is shown in table 10. In most β^0 thalassemias the β structural locus is also intact although in some β^0, as well as other rare β thalassemia states, partial or complete gene deletion is noted (49-51).

Unfortunately, the three most common forms of β thalassemia are associated with an intact β structural gene locus, at least within the limits of the present techniques. Thus, gene mapping or structural gene quantitation is infrequently utilizable for pre-natal diagnosis of the β thalassemias. It can be utilized in those rare circumstances where both parents are heterozygous for one of the deletion forms of β thalassemia (52). Recently, in the Sardinian population, where over 25% of pregnancies are at risk for homozygous β^0 thalassemia of a non-deletional type, a genetic polymorphism in the adjacent DNA 3' sequences has been observed (56). This will allow pre-natal diagnosis by restriction endonuclease techniques in approximately one-third of these pregnancies. The

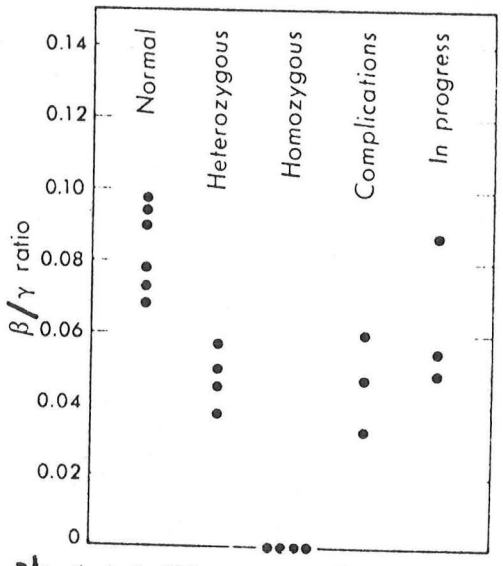
Table 10

Abnormalities Identified in the β Thalassemia States

Type	β Globin Synthesis	Quantity β-mRNA	β Gene Locus
β^+	Decreased	Decreased	Intact
β^o	Absent	Absent	Intact
β^o	Absent	Decreased	Intact
β^o	Absent	Absent	Partial deletion
$\delta^o\beta^o$	Absent	Absent	δ β deleted
$\delta^o\beta^o$	Absent	Absent	δ deleted β partial
$\delta^o\beta^o$	Absent	Absent	δ β deleted A γ deleted
Pancellular HPFH	Absent	Absent	δ β deleted
Lepore Hgb	Absent	Absent	δ β deleted

most widely applicable laboratory technique in the intrauterine diagnosis of homozygous β thalassemia has been that of globin chain synthesis. Fetal blood is obtained by fetoscopy or placental aspiration at about 17-23 weeks gestation. The procedure is successful in about 90% of cases, although multiple attempts may be necessary. The fetal blood is then incubated with radiolabelled amino acids and chain synthesis (β:γ) ratios are measured. In those cases aborted for the diagnosis of homozygous thalassemia or carried to term because of the diagnosis of normal or heterozygous state the prediction has been essentially 100% accurate (Figure 9). The primary drawback has been the difficulty of the fetal blood sampling procedures and a resultant fetal loss of approximately 10%. Nevertheless, this has been a significant advance in the clinical options available for this disease (53-55).

The alpha thalassemias are also a complex group of disorders at the molecular level and produce an even wider spectrum of clinical manifestations than do the β thalassemias. The latter circumstance is due to the fact that the α globin structured genes are reduplicated in the human (57). Thus each normal individual possesses a complement of four α structural genes. One of the population groups in which a high gene frequency of α thalassemia is recognized are Orientals. In persons of Chinese and Southeast Asian ancestry, the vast majority of the α thalassemia syndromes derive from deletions of one or both of the α structural genes on a given chromosome (58). Thus, clinical phenotypes will be expressed of four general types depending on the number of structural genes inherited (from 0-4) (59-60). The possible combinations and a summary of the clinical manifestations are shown in figure 10. The single



β/γ ratios in the 20 fetuses at risk of β-thalassæmia.

Diagnoses of "normal" and "heterozygous" were confirmed after birth. In the third and fourth columns, the findings were substantiated by study of fetal blood after abortion.

Figure 9
Ref. 53

Figure 10

Clinical Manifestations of Alpha Thalassemias

Gene Deletions	Clinical Picture
Single	Normal or Microcytosis
Double	Microcytic Anemia; A_2 and F normal.
Three	Microcytic and Hemolytic Anemia; A_2 Normal; F<5%, Hgb H
Four	Hydrops Fetalis; > 90% Barts Hgb.

gene deletion is termed α thalassemia - 2. A double deletion of the genes on one chromosone is called alpha thalassemia - 1. Hetrozygosity for both of these states produces the disorder, Hgb H disease and, as would be predicted, homozygosity for alpha thalassemia - 1 is incompatible with the survival of the fetus. This results from the fact that all known functioning hemoglobins possess a pair of α chains and a pair of non-α chains. Also, a clinical expression of the same type will result from either heterozygosity for α - thal - 1 or homozygosity for alpha thal -2 (1).

More extensive studies in other population groups have also revealed a significantly high incidence of the double gene deletion phenotype in persons of African and Mediterranean area origin. Nevertheless, in those racial groups, Hgb H disease is rather rare and hydrops fetalis has not been described (1). Recent studies of gene number and gene mapping utilizing cDNA hybridization techniques have clarified this apparent inconsistency. The α thal - 1 genotype is only rarely recognized in these population groups and therefore the double gene deletion phenotype is almost always homozygous α thal - 2. In those few patients with hemoglobin H disease in these population groups who have been investigated, the phenotype is of two general varieties. The three gene deletion form is the mechanism in ½ to 2/3 of the cases, but in the remainder, there are dysfunctional genes present similar to the events in the majority of β thalassemia types (Figure 11). Thus, although α thal - 2 is very common, α thal - 1 is rare and the likelihood of encountering homozygous α thal - 1 becomes vanishingly small (61-64).

Figure 11

Alpha Thalassemia Genotypes

Single Chromosome Gene Complement	Functional Behavior	Occurrence	
α α	Normal	All races	
α	Single locus	Asians	Common
		Blacks	25%
		Mediterranean	10-40%
—	Absent loci	Asians	Common
		Blacks	< 0.2%
		Mediterranean	Rare
α α Th	Single locus	Asians	Rare
		Blacks	Unknown
		Mediterranean	Common
α Th	< 1 locus	Asians	Unknown
		Blacks	Unknown
		Mediterranean	Common
α α cs*	Single locus	Asians	Uncommon
		Blacks	Unknown
		Mediterranean	Unknown

* CS = Hemoglobin constant spring

Hemoglobin H disease, although characterized by hemolytic anemia, is compatible with a nearly normal life expectancy and a lack of a significant incidence of symptomatic iron overload. Therefore, the primary defect that would be desirable to detect pre-natally would be homozygous α thalassemia - 1 ie. the four gene deletion or hydrops fetalis. This would allow therapeutic abortion preventing the medical and emotional consequences of carrying a dead fetus to full term. Fortunately, the only thalassemia defect so far detected that leads to this syndrome is a gene deletion syndrome. Thus simpler clinical techniques can, and have, been applied to pre-natal diagnosis in women at risk for having a fetus with this diagnosis. Since no α chain structural gene sequences will be detected in fetuses with this problem, the technique of gene mapping by DNA hybridization can be applied. This can be performed on any nucleated cell and thus has permitted the safer and simpler procedure of amniotic fluid sampling with harvesting and culturing of fetal fibroblasts for analysis. To date, such attempts have been uniformly successful and accurate (65-67).

Another clinical problem that has been clarified by these molecular genetic studies has been the explanation for a high frequency of microcytic states in black Americans which could not be explained by iron deficiency or β thalassemia. Between 5-10% of this population has such a finding. The studies of Dozy et. al. have demonstrated the prevalence of α -thalassemia 2 is 27.5% and homozygous alpha thalassemia-2 is 1.9% (61). Recent observations from this institution have demonstrated that 7.2% and 8.7% of black males who are not anemic with hemoglobin AA and AS respectively have microcytosis. The incidence in patients with hemoglobin AC is 15.2% (68). This suggests that the α- thalassemia - 2 heterozygous state is not always phenotypically silent and with certain abnormal hemoglobins the phenotypic expression is even more likely. This contrasts with the commonly accepted feeling that this condition is always clinically silent.

B. Conditions Effecting the Expression of Thalassemia - Potential for Genetic Modification

Another area of interest for potential manipulation in the thalassemia syndromes has been the consideration of altering the basic molecular abnormality to lessen the degree of clinical expression. A number of clinical examples of molecular alterations of the thalassemias which reduce the severity of these disorders are recognized. The basic feature of each of these circumstances is that the degree of alpha and non-alpha globin chain imbalance is reduced. An understanding of the molecular mechanisms involved in these events is being sought with the goal of attempting to manipulate hemoglobin synthesis in affected individuals along these lines. Table 11 lists a number of examples of such naturally occurring genetic disorders which appear to reduce the severity of the disorder that would be predicted by the molecular abnormality.

Homozygous β+ thalassemia in blacks has long been recognized to be less severe than the same molecular lesion in most other racial groups. Lesser degrees of globin chain imbalance, especially in the bone marrow, characterizes these individuals, although the mechanism for this is not clear (69, 70). The co-existence of alpha thalassemia might be expected to reduce the level of globin chain imbalance, and indeed, certain patients who are homozygous for β^{o} or severe β+ thalassemia appear to have a modulation in the clinical severity of their disease (thalassemia intermedia) on this basis (71). Another observation which would

Table 11 Conditions Modifying the Expression of Imbalanced Globin Chain Synthesis

Homozygous β+ thalassemia in blacks

Homozygous β thalassemia plus α thalassemia

δ^{o} β^{o} thalassemia

Pancellular hereditary persistence of fetal hemoglobin

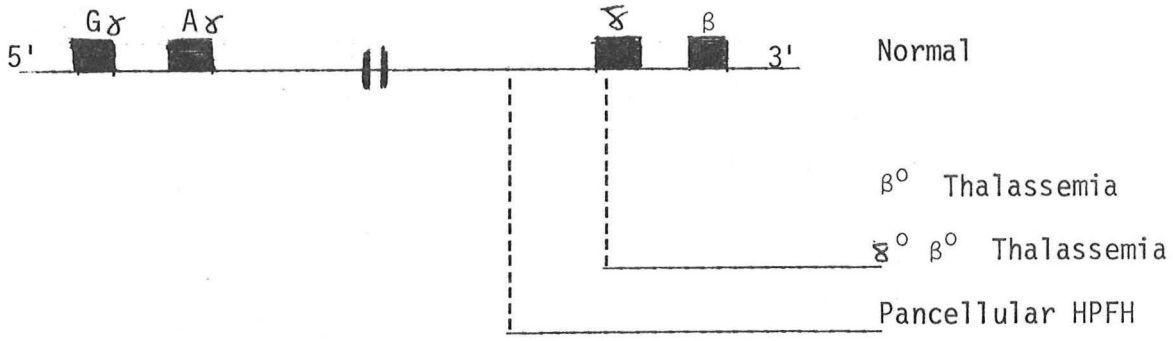
Heterocellular hereditary persistence of fetal hemoglobin

support the meliorating effect of α thalassemia on homozygous β thalassemia expression is the observation that the frequency of homozygous α thal -2 in Mediterraneans with homozygous β thalassemia is over 6x that of non-β thalassemics in the same population (72). Homozygosity for common β^{o} thalassemia, δ^{o} β^{o} thalassemia and pancellular hereditary persistence of fetal hemoglobin (HPFH) produce clinical syndromes of severe, moderately severe (thalassemia intermedia) and minimal degree respectively (1) (Table 12). In all three circumstances, no

Table 12 Clinical Manifestations of Homozygous Thalassemia Syndromes

	Anemia	% Hgb A	% Hgb A_2	% Hgb F	Chain Imbalance
β^{o} Thalassemia	Major	0	2-5	95-98	Severe
δ^{o} β^{o} Thalassemia	Intermedia	0	0	100	Mod severe
Pancellular HPFH	None	0	0	100	Slight

demonstrable β chain m-RNA is produced in the homozygote (36). Nevertheless α and non-α globin chain synthesis is imbalanced to the degree predicted by the clinical severity. The moderate degree of imbalance in δ^{o} β^{o} thalassemia and the minimal imbalance in pancellular HPFH are due to greater degrees of γ chain synthesis in these two disorders compared to β^{o} thalassemia (36). Extensive gene mapping utilizing restriction endonucleases have characterized, in a major way, the genetic alterations in these three disorders (Figure 12). The entire γδβ gene complex appears to be intact in most β^{o} thalassemia. In δ^{o} β^{o} thalassemia, a deletion beginning in the 5' region of the δ gene and extending beyond the 3' end of the β gene has occurred. In pancellular HPFH the deletion begins in the intergenic region between the Aγ and δ genes and extends through and beyond the β gene (50). This has led to the hypothesis that some of the sequences in the Aγ-δ intergenic region are important in the regulation of γ to β chain switching and the transcription of γ chain sequences. It would be hoped that an understanding of these events might allow manipulation of γ chain synthesis in β thalassemia to reduce chain imbalance.



Solid lines represent deleted gene regions

Figure 12
Ref. 36

Recently, an example of an *in vitro* alteration of a thalassemic defect allowing expression of β chain synthesis has been described. Chang and Kan described a β^{o} thalassemia in which a nonsense mutation at position 17 of the beta gene produced a terminator codon (46). In a cell free system, no β chain is synthesized although the altered β mRNA is present. However, addition of a serine suppressor t-RNA to the system resulted in the production of a new polypeptide chain which had the characteristics of a β globin chain with serine inserted at the 17 position (83). If other β^{o} thalassemias also are found to be consequences of similar nonsense mutations, comparable manipulations at an *in vivo* level are potentially possible and might provide a means of ameliorating the consequences of globin chain imbalance.

An even more intriguing possibility exists in the circumstance of heterocellular HPFH. Hemoglobin F predominates in fetal life until approximately 36 weeks of gestation when β chain synthesis begins to increase such that by the age of 1-2 years less than 2% of the total hemoglobin pool is Hgb F (73). Throughout fetal life and post-natal life, the hemoglobin F is present in cells in a heterogenous distribution. With normal adult levels, the small amount of Hgb F (< 2%) is found in approximately 0.1 - 8% of erythrocytes, termed "F cells" although these cells also contain Hgb A (74, 75). There is no evidence that these cells represent a specific clone of erythrocytes (76). In fetal life, pregnancy, many anemias and the β thalassemias, Hgb F levels are often slightly increased and this is due to an increase in the number of F cells rather than a uniform increase in Hgb F in all cells (74, 75). In the thalassemias, it appears that the increase in Hgb F proportions is due to a selective survival advantage of these cells presumably because the chain imbalance in them is less (78). In pancellular HPFH, the increase in hemoglobin F is homogeneously distributed in all cells, thus explaining the near normalcy of persons with this condition (77). Recently, a genetically determined disorder has been described in several population groups which is associated with a 3-5 fold increase in hemoglobin F concentration due to an increase in the number of Hgb F cells (heterocellular HPFH) (79). Of pertinence to this discussion, persons who are heterozygous for this disorder and heterozygous for β thalassemia have higher

hemoglobin F levels (more Hgb F cells) than either the heterozygotes for HPFH alone or their siblings heterozygous for β thalassemia only (80). It has been predicted that an individual homozygous for severe thalassemia and also possessing this genetic disorder would have a milder degree of severity than would be predicted. Such an individual with this probable genotype has been observed and studied at this institution (81, 92). This 45 year old woman has clinical, biochemical and family study evidence of homozygous β^o thalassemia. In addition, the maternal side of the family appears to also possess a gene for heterocellular hereditary persistence of fetal hemoglobin being inherited in linkage with the thalassemia defect. The proband therefore has inherited this form of HPFH with her homozygous thalassemia and has a non-transfusion dependent type of thalassemia intermedia rather than the predicted lethal state of homozygous β^o thalassemia.

The characterization of the mechanisms of heterocellular HPFH is of extreme interest since the ability to increase the production of Hgb F cells would hopefully be a means of ameliorating the clinical manifestations of homozygous β thalassemia.

In summary, the past decade has been characterized by significant advances in the management of severe homozygous thalassemia which will hopefully lead to both a longer lifespan and better quality of life for these individuals. In addition, detailed analyses of the molecular genetics of hemoglobin synthesis and thalassemia have occurred as a consequence of innovative basic science technology. A byproduct of these molecular techniques has been the beginning applicability to alternate approaches of attack such as pre-natal diagnosis. Perhaps the next decade will be characterized by the development of techniques of genetic engineering which can be applied to this and other hereditary molecular disorders.

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