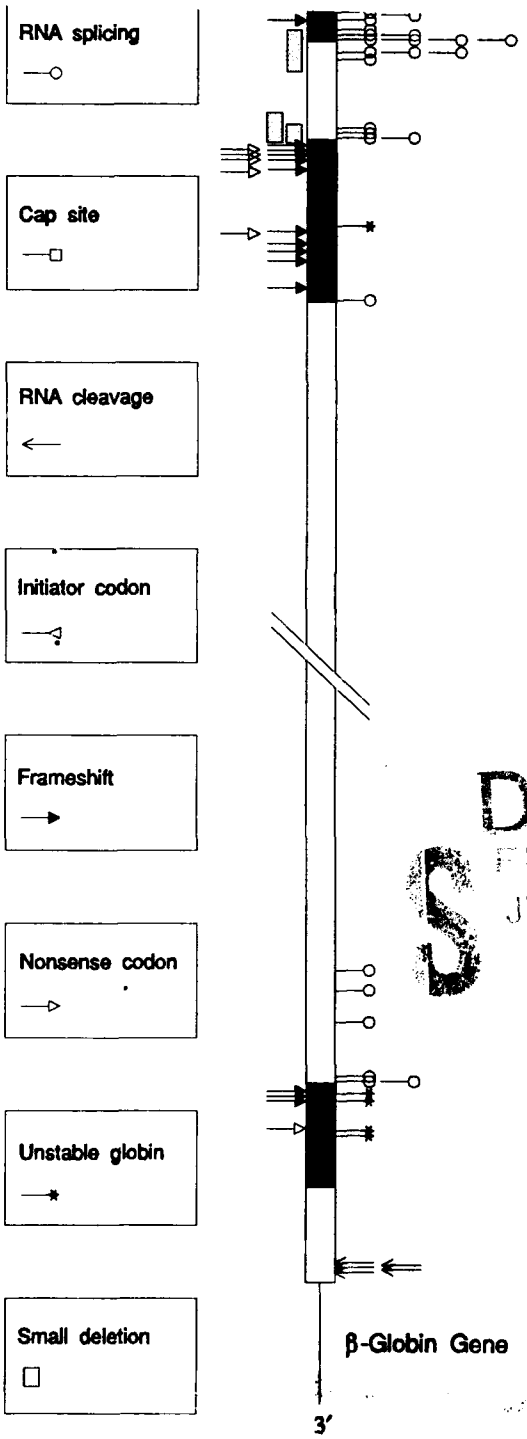

Sixth Cooley's Anemia Symposium

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Editor
Arthur Bank

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Edited by Arthur Bank

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SIXTH COOLEY'S ANEMIA SYMPOSIUM[a]

Editor and Conference Organizer
ARTHUR BANK

CONTENTS

[a] This volume contains papers from a conference entitled Sixth Cooley's Anemia Symposium, which was cosponsored by the New York Academy of Sciences and the Cooley's Anemia Foundation, Inc., and held in New York on March 13–15, 1990.

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Part III. Antenatal Diagnosis

Part IV. Therapy of Thalassemia

Part V. Future Research in Thalassemia

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Preface

This volume contains the papers from the Sixth Cooley's Anemia Symposium. Over the past 25 years, remarkable progress has been made in our understanding of the pathogenesis of molecular defects in this disease. Since the last symposium six years ago, there have been vastly improved diagnostic tests and several new approaches to the treatment of Cooley's anemia. The data reported in this volume include a summary of advances to date in our knowledge of (1) the molecular defects in the thalassemias, (2) the regulation of fetal hemoglobin production, (3) antenatal diagnosis of thalassemia, (4) iron chelation therapy, (5) bone marrow transplantation, (6) gene therapy for the thalassemias, and (7) psychosocial problems of thalassemic patients and their families. All these topics are discussed by experts from around the world, and from different points of view. It was the goal of this symposium to have a sharing of ideas between scientists from different countries and interested physicians. In addition, this is the first symposium to discuss in detail the psychosocial consequences of Cooley's anemia on patients and their families. We hope this volume provides a comprehensive view of Cooley's anemia from the perspective of the information available in 1990.

I would especially like to thank the Cooley's Anemia Foundation for their efforts in support of this symposium. I dedicate this volume to Mr. Robert Ficarra of the Cooley's Anemia Foundation because of the time and energy he has donated in his unwavering support of Cooley's anemia research over the past several years. I would also like to thank Dr. David Badman of the NIDDKD, and Drs. Alan Levine and Helena Mishoe of the NHLBI, for their moral and financial support and their continued dedication to Cooley's anemia research. I also owe particular gratitude to Dr. Maria Simpson and Ms. Renée Wilkerson of the New York Academy of Sciences, and Ms. Oona Collins, my Executive Assistant at Columbia University, for all their help in organizing this symposium. Lastly, I thank my colleagues in the United States and abroad who made the symposium a most fulfilling event.

ARTHUR BANK

PART I. GENETIC DEFECTS IN THALASSEMIAS AND RELATED DISORDERS

Gene Defects in β-Thalassemia and Their Prenatal Diagnosis

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MOLECULAR BASIS OF β-THALASSEMIA

In contrast to α-thalassemia, β-thalassemia is generally caused by point mutations. These mutations were studied extensively from 1980 to 1986, usually by haplotype analysis of the β-globin gene cluster followed by cloning and sequencing of the mutant β-globin gene. About 40 point mutations producing β-thalassemia were discovered by mid-1987, and many were documented by transient expression studies of the mutant gene.[1]

In 1987 it became possible to amplify regions of the β-globin gene starting with genomic DNA from leukocytes[2,3] and to sequence directly the amplified products.[4,5] By use of this approach in selected individuals known to lack the common alleles of the ethnic group represented by the patient under study, about 50 more alleles have been characterized in less than 2 years.[6] In July 1990, the total number of point mutations which produce β-thalassemia known to the authors is 91 (TABLE 1 and FIG. 1). Since the total number of β-globin alleles known to produce any clinical phenotype is roughly 200, the β-thalassemia alleles now account for over 45% of the clinically significant alleles at the β-globin locus, and the "abnormal hemoglobins" account for the remainder.

The spectrum of β-thalassemia alleles has been determined in a wide variety of population groups, including American blacks,[7,8] Asian Indians,[9,10] Italians and Greeks,[11] Spanish,[12] Turks,[13] Kurdish Jews,[14] Chinese,[15,16] Japanese,[17] and Thai,[18] among others. In most affected population groups, such as Mediterranean peoples, Chinese and Southeast Asians, Asian Indians, and blacks of African origin, a handful of ethnic-group-specific alleles accounts for roughly 90–93% of β-thalassemia genes. For example, among Chinese from Kuontung province, 4 alleles account for about 90% of β-thalassemia genes.[15] On the other hand, a large number of rarer alleles have been observed in each ethnic group. For example, to date 11 rare alleles account for the remaining 10% of β-thalassemia genes in Chinese. The total number of mutations observed in the well-studied groups is, in Chinese, 15; Asian Indians, 10; blacks, 12; Mediterraneans, 31. In the broad group of Mediterranean peoples it is important to note that although many of the 31 alleles have been observed in diverse regions of the Mediterranean basin, allele frequencies vary greatly from one country to another. Because so many alleles are found in each region, most individuals with β-thalassemia major carry two different alleles and are called genetic compounds.

True homozygotes who carry two copies of the same allele are in the minority. Because a wealth of general information about nucleotide sequences important for gene expression is derived from the paradigm of the β-thalassemia mutations, a brief discussion of the molecular effects of these mutations follows.

TRANSCRIPTION MUTANTS

A large number of alleles which affect transcription have been observed. These have provided new information on critical nucleotide sequences of specialized genes beyond that of *in vitro* transcription studies. The mutations are concentrated in the TATA box (the sequence CATAAAA located roughly 30 nucleotides upstream of the cap site) and in the proximal and distal CACACCC sequences at −90 and −105 nucleotides (nts) upstream of the gene.

In vitro transcription studies had previously implicated the TATA region's importance for proper efficiency of transcription and the location of the start site of transcription.[19] Mutations are now known at the ATAA residues at nts −31, −30, −29, and −28. These mutations are generally associated with mild clinical phenotypes and with reduced transcription beginning at the appropriate nt, +1. However, ethnic variation in phenotype, probably due to modifier sequences, is observed. Black homozygotes with the −29 A → G mutation show very mild symptoms[7] or are even silent for β-thalassemia, while the single known Chinese homozygote for the −29 mutation has transfusion-dependent thalassemia major.[20] The explanation for this difference in clinical phenotype is believed to lie in the presence or absence of a partial up-promoter substitution (C → T) at −158 to the $^{G}\gamma$ globin gene. This up-promoter substitution is present in the chromosome bearing the Black −29 mutation, but it is absent from the chromosome carrying the Chinese −29 mutation.[20]

A large number of relatively mild mutations occur in the proximal CACACCC sequence at −90. These are located at the 5′ C at −92 and at the 3′ C residues at nts −88, −87, and −86 (See TABLE 1). A total of five different substitutions have now been observed in this region. The −92 mutation has not been proven by transient expression studies. A "silent carrier" mutation has been found in the distal CACACCC sequence at the 3′ C residue at −101.[21] Only a single carrier each is known for the −92 and −86 (C → G) mutations and these two individuals have MCVs (mean corpuscular volumes) of 85 and 70, and Hb (hemoglobin) A_2 values of 4.5% and 3.9%, respectively. The −86 carrier has moderate β-thalassemia trait and was detected by routine screening. Routine screening without Hb A_2 determination would not have detected the individual carrying the −92 mutation.

It is of great interest that even though 12 transcriptional mutations are now known, none have yet been found in the CCAAT box at −70 from the cap site. The CCAAT box was one of the first regions to be implicated in regulatory activity *in vitro* studies of promoter activity using the β-globin gene.[22] Whether this region has importance in transcription regulation *in vivo* is still unclear.

RNA MODIFICATION MUTANTS

Cap Site Mutant

The +1 nucleotide is the start site for transcription and also the site at which 5′ end modification (capping) of precursor RNA occurs. However, it is clear from a

TABLE 1. Point Mutations in β-Thalassemia[a]

Mutant Class	Type[b]	Origin
Nonfunctional mRNA		
Nonsense mutants		
Codon 17 (A→T)	0	Chinese
Codon 39 (C→T)	0	Mediterranean, European
Codon 15 (G→A)	0	Asian Indian
Codon 121 (G→T)	0	Polish, Swiss
Codon 37 (G→A)	0	Saudi Arabian
Codon 43 (G→T)	0	Chinese
Codon 61 (A→T)	0	Black
Codon 35 (C→A)	0	Thai
Codon 22 (G→T)	0	Reunion Islander
Frameshift mutants		
−1: codon 1 (−G)	0	Mediterranean
−2: codon 8 (−AA)	0	Turkish
−1: codon 16 (−C)	0	Asian Indian
−1: codon 44 (−C)	0	Kurdish
+1: codon 47 (+A)	0	Surinamese black
+1: codons 8/9 (+G)	0	Asian Indian
−4: codons 41/42 (−CTTT)	0	Asian Indian, Chinese
−1: codon 6 (−A)	0	Mediterranean
+1: codons 71/72 (+A)	0	Chinese
+1: codons 106/107 (+G)	0	American black
−1: codons 76 (−C)	0	Italian
−2: codon 5 (−CT)	0	Mediterranean
−1: codon 11 (−T)	0	Mexican
−1: codon 35 (−C)	0	Indonesian
−2, +1: codon 114 (−CT, +G)	+	French
+1: codons 14/15 (+G)	0	Chinese
−7: codons 37–39	0	Turkish
+2: codon 94 (+TG)	0	Italian
−1: codon 64 (−G)	0	Swiss
−1: codon 109 (−G)	+	Lithuanian
−1: codons 36–37 (−T)	0	Kurdish (Iranian)
+1: codons 27–28 (+C)	0	Chinese
+1: codon 71 (+T)	0	Chinese
−1: codons 82/83 (−G)	0	Azerbaijani
−1: codon 126 (−T)	+	Italian
−4: codons 128–129, −11: codons 132–135, +5: codon 129	+	Irish
Initiator codon mutants		
ATG→AGG	0	Chinese
ATG→ACG	0	Yugoslavian
RNA Processing Mutants		
Splice junction changes		
IVS-1 position 1 (G→A)	0	Mediterranean
IVS-1 position 1 (G→T)	0	Asian Indian, Chinese
IVS-2 position 1 (G→A)	0	Mediterranean, Tunisian, American black
IVS-1 position 2 (T→G)	0	Tunisian
IVS-1 position 2 (T→C)	0	Black
IVS-1 3′ end (−17 bp)	0	Kuwaiti
IVS-1 3′ end (−25 bp)	0	Asian Indian

NOTE: Table continues on following two pages.

TABLE 1 (continued).

Mutant Class	Type[b]	Origin
RNA Processing Mutants (*continued*)		
Splice junction changes (*continued*)		
IVS-1 3′ end (G→C)	0	Italian
IVS-2 3′ end (A→G)	0	American black
IVS-2 3′ end (A→C)	0	American black
IVS-1 5′ end (−44 bp)	0	Mediterranean
IVS-1 3′ end (G→A)	0	Egyptian
Consensus changes		
IVS-1 position 5 (G→C)	+	Asian Indian, Chinese, Melanesian
IVS-1 position 5 (G→T)	+	Mediterranean, black
IVS-1 position 5 (G→A)	+	Algerian
IVS-1 position 6 (T→C)	+	Mediterranean
IVS-1 position −1 (G→C) [codon 30]	+	Tunisian, black
IVS-1 position −1 (G→A) [codon 30]	?	Bulgarian
IVS-1 position −3 (C→T) [codon 29]	?	Lebanese
IVS-2 3′ end (CAG→AAG)	+	Iranian, Egyptian, black
IVS-1 3′ end (TAG→GAG)	+	Saudi Arabian
IVS-2 3′ end position −8 (T→G)	+	Algerian
Internal IVS changes		
IVS-1 position 110 (G→A)	+	Mediterranean
IVS-1 position 116 (T→G)	0	Mediterranean
IVS-2 position 705 (T→G)	+	Mediterranean
IVS-2 position 745 (C→G)	+	Mediterranean
IVS-2 position 654 (C→T)	0	Chinese
Coding region substitutions affecting processing		
Codon 26 (G→A)	E	Southeast Asian, European
Codon 24 (T→A)	+	American black
Codon 27 (G→T)	Knossos	Mediterranean
Codon 19 (A→G)	Malay	Malaysian
Transcriptional Mutants		
nt −101 (C→T)	+	Turkish
nt −92 (C→T)	+	Mediterranean
nt −88 (C→T)	+	American black, Asian Indian
nt −88 (C→A)	+	Kurdish
nt −87 (C→G)	+	Mediterranean
nt −86 (C→G)	+	Lebanese
nt −31 (A→G)	+	Japanese
nt −30 (T→A)	+	Turkish
nt −30 (T→C)	+	Chinese
nt −29 (A→G)	+	American black, Chinese
nt −28 (A→C)	+	Kurdish, Mexican
nt −28 (A→G)	+	Chinese
RNA Cleavage and Polyadenylation Mutants		
AATAAA→AACAAA	+	American black
AATAAA→AATAAG	+	Kurdish
AATAAA→A (−AATAA)	+	Arab
AATAAA→AATGAA	+	Mediterranean
AATAAA→AATAGA	+	Malaysian

TABLE 1 (continued).

Mutant Class	Type[b]	Origin
	Cap Site Mutants	
nt +1 (A→C)	+	Asian Indian
	Unstable Globins	
$\beta^{Indianapolis}$ (codon 112; Cys→Arg)	+	European
$\beta^{Showa\text{-}Yakushiji}$ (codon 110; Leu→Pro)	+	Japanese
$\beta^{Houston}$ (codon 127; Gln→Pro)	+	British
Codons 127–128 (−AGG; Gln, Ala→Pro)	+	Japanese
Codon 60 (Val→Glu)	+	Italian

[a]The total number of point mutations (91) known as of July 1990 is listed. For references, see review in Ref. 55.
[b]0, β^0; +, β^+; E, β^E; Knossos, $\beta^{Knossos}$; Malay, β^{Malay}; ?, unknown.

number of genes that any nucleotide, not just the commonly seen A residue, can serve as the site of RNA capping. The observed A → C mutation produces very mild symptoms; a homozygote has the hematologic values of a mild β-thalassemia carrier, and heterozygotes have low normal MCV values and borderline normal Hb A_2 levels.[5] This mild mutation may act on transcription (reduced β-globin mRNA is observed after *in vitro* gene transfer of a β-globin gene bearing an altered +1 nucleotide[23]) or on capping with a secondary effect on translation. Experiments to distinguish between these possibilities are very difficult to accomplish and have not yet been carried out.

It is of interest that the combination of this mutation, which is nearly a silent carrier allele, with a severe β-thalassemia allele can produce β-thalassemia major.[5] We have observed transfusion-dependent thalassemia in two unrelated children with this β-globin genotype (C. D. Boehm, unpublished observation).

RNA Cleavage and Polyadenylation Mutants

At the 3′ end of eukaryotic genes is a signal sequence AATAAA, which in the precursor RNA is AAUAAA. Ten to twenty nucleotides 3′ to this sequence is the site of cleavage of the nascent RNA transcript and the point to which a polyadenylic acid [poly(A)] tail is added. In the AATAAA sequence, 4 different nucleotide substitutions and a 5-nt deletion have been seen to date (see TABLE 1). Two of these five mutants have been studied by transient expression analysis. In both cases only a small percentage of the RNA transcript is cleaved appropriately and nearly all transcripts are not cleaved until transcription proceeds beyond AATAAA signals one to three kilobases 3′ to the gene.[14,24] Since the concentration of these elongated transcripts *in vivo* is about 10% of that expected, it is presumed that deficient β-globin synthesis associated with these mutants is secondary to instability of the abnormal elongated transcripts. All mutations of this type are β^+-thalassemic alleles, because they produce some normal transcripts. Also, it is likely that normal β-globin is synthesized from any elongated transcripts present *in vivo*.

Mutants Affecting RNA Splicing

Critical sequences in RNA splicing lie at the exon-intron splice boundaries. The splicing process begins with cleavage of the 5′ splice junction and a looping back of the 5′ end of the intron to form a covalent 5′–2′ phosphodiester bond with an A residue 30 or so nucleotides 5′ to the 3′ splice junction.[25,26] The resulting structure is called a lariat. The first two nucleotides at both the 5′ and the 3′ end of the intron are essential for splicing. Nearly all eukaryotic genes have a GT sequence at the 5′ end and an AG sequence at the 3′ end of every intron. To date, 10 different mutations are known to alter these GT or AG residues; they all produce β^0-thalassemia alleles.

Other key sequences at the splice junctions are also important for efficient splicing. After a large number of eukaryotic genes were sequenced, marked similarities in sequence were seen at many intron-exon boundaries. These identified sequences were called consensus sequences. For donor sites (5′ end of introns) they

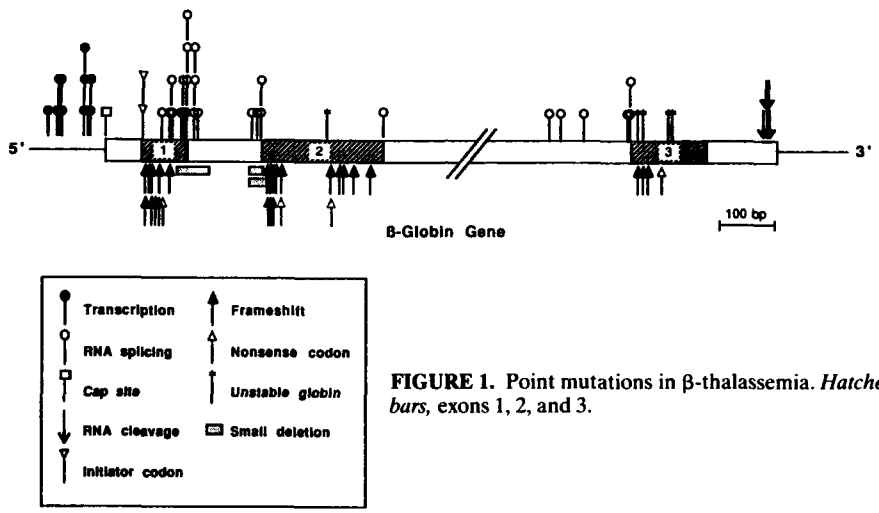

FIGURE 1. Point mutations in β-thalassemia. *Hatched bars,* exons 1, 2, and 3.

encompass the last 3 nucleotides of the exon and the first 6 nucleotides of the intron; for acceptor sites (3′ end of introns) they are the last 10 nucleotides of the intron and the first nucleotide of the exon.[27] Seven consensus changes have been observed in donor sites of β-globin genes, all in the donor site of intron 1. They occur at nts −3, −1, +5, and +6 from the splice site. The mutation at +6 leads to an especially mild allele, while mutations at +5 (all three possible nucleotide substitutions have been seen) produce diverse clinical phenotypes.

At the acceptor site, at position −3 from the splice junction, mutations in which the consensus nucleotide, a pyrimidine, is changed to a purine have been observed in both intervening sequence (IVS) 1 and IVS-2.[5,8] Another mutation in the consensus sequence for acceptor sites substitutes an A for a C in the polypyrimidine tract 8 nts 5′ to the splice site.[28] It is curious that no other mutations have been observed in this tract in either intron even though the polypyrimidine tract is at least 6 nucleotides long in the consensus sequence.

Nucleotide substitutions can lead to the presence of a consensus splice site sequence within either an intron or an exon (FIG. 1). These mutations are very interesting because their location and nature need to be explained in any proposed mechanism of splicing. In intron 2, mutations of this type produce new donor sites roughly 100, 150, and 200 nts from the acceptor site of IVS-2.[29] The first two lead to reduced production of normally spliced RNA, while the latter eliminates normal splicing and is a β^0-thalassemia allele. Mutations of this type in exon 1 activate the cryptic splice site in codons 24–27 and are generally mild alleles. A cryptic splice site is a sequence which mimics the consensus sequence for a splice site but is never used under normal circumstances. When a mutation in a cryptic site, e.g., codons 24–27, makes the site resemble more closely the normal splice site, the mutant site becomes no longer cryptic and it is used at a low efficiency for splicing.

One of these codon-26 mutations in exon 1, the β^E allele, leads to deficient production of β^E-globin, which contains a Glu → Lys substitution at amino acid 26.[30] The abnormal-globin thalassemia phenotype is due to production of two forms of β-globin mRNA. A low level of normally spliced mRNA contains the exon-1 substitution, leading to deficient production of β^E-globin. For the remainder of the mRNA, splicing into the codons-24–27 site does not lead to a recognizable β-globin. The β^E gene, with its high prevalence in Southeast Asia, is probably the second most common abnormal hemoglobin gene in the world. In all, about 35% (31 of 91) of the known mutant alleles affect RNA splicing in one of the ways outlined.

TRANSLATION MUTANTS

Thirty-seven of the 91 known mutant alleles affect translation of the RNA into globin. Nine of these are nonsense codons which terminate translation. Twenty-six more are frameshifts due to insertion or deletion of 1, 2, 4, or 7 nucleotides in one of the exon sequences coding for β-globin. Two are substitutions in the initiator codon for translation, which is ATG in the DNA. These two recently observed initiator codon mutations, both of which are rare, affect the same nucleotide: ATG → AGG and ATG → ACG (see TABLE 1). Of the translation mutations, 3 alleles (nonsense codon 121, nonsense codon 39, and frameshift 64) have occurred as *de novo* mutations.[31–33]

DELETIONS PRODUCING β-THALASSEMIA

A number of deletions affecting much of the β-globin gene cluster have been observed (see Ref. 33a for review). Many of these in which both δ- and β-globin genes are deleted give rise to hereditary persistence of fetal hemoglobin (HPFH) and δβ-thalassemia. A handful of deletions, all but one of which are rare, affects only the β-globin gene and produces β-thalassemia.[34–39] The common deletion removes 619 bp of IVS-2, exon 3, and sequences 3′ of the β-globin gene.[39] It accounts for about one-third of the β-thalassemia genes among Asian Indians.[9]

Three other deletions are of particular interest because they leave the β-globin gene intact and yet silence its expression. They are all rare, having been reported only in heterozygotes, and they have their 3′ ends 2.5 kb, 30 kb, and 50 kb 5′ of the β-globin gene.[40–42] These deletions cause so-called position effects, which (as previously defined by *Drosophila* geneticists) involve the variable expression of a particular gene that is dependent on the location of that gene in the genome. The deletions

cited above eliminate the 5′ end of the β-globin gene cluster and ≥30 or kb 5′ to the cluster. One could hypothesize that these deletions silence the β-globin gene either (1) by bringing chromatin into the cluster that is "closed," thereby blocking transcription of neighboring gene sequences or (2) by eliminating DNA sequences 5′ to the cluster which are important in activating transcription of all genes in the cluster at the appropriate developmental stage. We now know that the second of these hypotheses is correct; the deletions silence the β-globin gene because they delete a "locus-activating" region.[43]

SILENT AND DOMINANT β-THALASSEMIA ALLELES: TWO ENDS OF A SPECTRUM

Over the past year we have learned more about the phenotypic variation associated with a number of β-thalassemic alleles. We now recognize that a spectrum of β-thalassemia alleles exists, all the way from silent carrier alleles to those that produce β-thalassemia intermedia in the heterozygous state. We also realize that comments on phenotypic variation are generalizations, and that while the phenotype associated with many of the 91 alleles is generally uniform, it may vary significantly in response to other modifying genetic factors. Two such modifying factors are the presence of α-thalassemia (silent carrier or trait), which may reduce the clinical severity of β-thalassemia, and an up-promoter substitution at −158 of the $^{G}\gamma$ gene, which may also ameliorate the disease.

Among the mild alleles are those that affect transcription, alter the cap site, activate cryptic splice sites, and disrupt consensus sequences for splicing. At the other end of the spectrum are more severe alleles that produce either a thalassemia intermedia or an inclusion-body hemolytic anemia in the heterozygous state. In general, these alleles produce a highly unstable β-globin which has a half-life measured in minutes.

Missense mutations producing amino acid substitutions at residues 110, 112 and 127 and a one-codon deletion at codon 127, leading to replacement of glutamine/alanine by proline, usually produce a thalassemia intermedia phenotype. Globin chain synthesis studies of the codon-110 mutation ($\beta^{Indianapolis}$) and the codon-127 mutation ($\beta^{Houston}$) suggest that these globins assemble weakly into $\alpha_1\beta_1$ dimers and that mixed $\alpha_2\beta^A\beta^X$ tetramers form and are rapidly degraded.[44,45]

The chronic hemolytic anemia phenotype results from more extensive changes to exon 3. Single-nucleotide deletions in exon 3 lead to an elongated β-globin of 156 amino acids and an altered amino acid sequence COOH-terminal to the frameshift mutation. Two mutations of this type have been seen; −2, +1 at codon 114[46] and −1 at codon 109. Heterozygosity for nonsense codon 121 also produces this phenotype.[47] The −1 frameshift in exon 3 leads to new proline residues at positions 115, 123, 129, 140, and 142. Nonsense codon 121 eliminates all residues COOH-terminal to amino acid 120. These mutations would completely disrupt the COOH-terminal region of β-globin and eliminate α_1-β_1 dimer assembly. β/α synthesis studies suggest that these β chains are highly unstable and, in contrast to $\beta^{Indianapolis}$ and $\beta^{Houston}$, do not participate in hemoglobin tetramers.

The genetic implications associated with these more severe mutations are noteworthy. These exon-3 mutant alleles are all very rare. Indeed, each one has been observed in single families from widely dispersed ethnic groups (French, Lithuanian, European, Japanese,[2] British, and Italian). It is postulated that the clinical phenotype associated with these alleles is sufficiently severe to counterbalance any reduced morbidity vis-á-vis malaria in heterozygotes. Lack of positive selection due to malaria

can easily explain the disseminated geographic distribution and low gene-frequency of these mutations.[45]

β-THALASSEMIA DUE TO UNKNOWN MUTATIONS

In 1984, Semenza *et al.* reported the presence in an Albanian of a silent carrier β-thalassemia mutation not linked to the β-globin gene cluster.[48] The mutation had been passed from a parent to two offspring, who received different β-globin gene clusters from that parent. Transient expression studies and sequence analysis showed no abnormality in the β-globin genes of the silent carrier parent. This β-thalassemia allele is presumably a mutation in another gene located elsewhere in the genome which is important in β-globin gene expression.

Over the past two years we have sequenced a large number (100) of β-globin genes, searching for novel β-thalassemia mutations. In 9 individuals who either had hematologic changes suggestive of β-thalassemia trait or had an affected child, we failed to find a causative mutation. In these instances we have analyzed the nucleotide sequence from 600 nucleotides 5′ of the cap site of the β-globin gene to 200 nts 3′ of the gene. In addition, we have sequenced the 3′ enhancer region from 700 to 1100 nts 3′ of the gene. We consider these β-thalassemia genes to be true unknowns and believe that a small fraction of β-thalassemia genes represent mutations at some distance from the β-globin gene. Perhaps some of these mutations are in the "locus-activating" region 5′ to the ε-globin gene. However, we have recently sequenced a key portion of this region in a number of our patients with unknown mutations and have not found any sequence alterations (J. Katz and H. H. Kazazian, unpublished observations).

PRENATAL DIAGNOSIS OF β-THALASSEMIA: 1990

In 1990 our prenatal diagnosis procedure begins by verifying that both members of the consulting couple are β-thalassemia carriers. This is done by obtaining documentation of red cell indices and hemoglobin A_2 levels on both prospective parents. In most instances, the consulting couple will not have borne an affected child previously, so we will obtain a blood sample from each prospective parent and learn the ethnic background of the couple. In the laboratory we then determine the nature of their β-thalassemia mutations. Over the past year we have carried out 90 prenatal diagnoses for β-thalassemia; 37 diagnoses were in couples who had undergone a previous prenatal diagnosis, but of the 53 newly referred couples only 11 (20%) previously had an affected child. Thus, the vast majority of newly referred couples have not dealt with raising a child afflicted with β-thalassemia.

In North America, all prenatal diagnosis of β-thalassemia is now carried out by direct detection of the causative mutation after polymerase chain reaction (PCR) amplification of the β-globin gene.[6,49,50] At Johns Hopkins University most diagnoses are made using hybridization of ^{32}P end-labeled oligonucleotides to an amplified β-globin gene region dotted on a nylon membrane. Because the β-globin sequence of interest has been amplified more than 10^6-fold, hybridization time can be limited to 1 h, and the entire procedure of DNA dotting, hybridization, washing, and film exposure is often carried out in 2 h. When a mutation either creates or obliterates an endonuclease site, one can digest the amplified product with that particular endonuclease, subject the digested product to agarose gel electrophoresis in a minigel, and

visualize the band pattern under ultraviolet light after ethidium bromide staining of the DNA. The presence or absence of digestion then signals the presence or absence of the mutation under investigation. This type of procedure is useful in detection of 5 of 7 common Mediterranean alleles and the β^S-globin gene but of few of the β-thalassemia alleles of other groups. Note that neither hybridization nor radioactive material is required in this procedure.

The common 619-bp deletion in Asian Indians is detected by amplification of a fragment which includes the deletion region. After gel electrophoresis of the amplified region, a 1215-bp fragment is produced when the deletion is present and a 596-bp fragment is derived from any β-globin gene lacking the deletion.

Occasionally, after screening both members of a couple for the mutations known to occur in their ethnic group, the cause of β-thalassemia remains unknown in one member. At that point our practice is to amplify by PCR the regions of the β-globin gene known to contain β-thalassemia mutations and to carry out direct sequence analysis of these regions. This procedure is nearly always successful in uncovering any unknown mutation. We then analyse fetal DNA for the presence of this mutation by sequencing the same region in amplified fetal DNA.[4,5] Rarely, the mutation remains unknown after sequence analysis. We then obtain blood samples from the parents of the individual carrying the unknown mutation and rapidly haplotype his/her β^A and β-thalassemia genes. For rapid haplotyping, we use multiplex amplification of 5 regions in the β-globin gene cluster followed by digestion of the products and minigel electrophoresis.[51] This haplotype approach using PCR will nearly always provide a marker for the β-thalassemia gene and a rapid diagnosis.

By use of PCR amplification followed by dot hybridization and/or restriction endonuclease digestion of amplified product, prenatal diagnosis is nearly always available within 3–7 days of fetal sampling. When parental blood samples are obtained prior to fetal sampling, one can discover the parental mutations in advance and provide fetal diagnosis within 2–3 working days. Thus, PCR techniques reduce the time required for diagnosis, and they increase accuracy by allowing direct mutation analysis.

Kan and colleagues have used a non-radioactive approach developed at Cetus Corporation to carry out prenatal diagnosis for β-thalassemia in Chinese couples.[52,53] This approach utilizes horseradish peroxidase labeling of the 5′ end of the oligonucleotides used for detection of mutations. The presence of an oligonucleotide hybridizing to amplified DNA is detected by histochemical staining for the presence of the enzyme.

A new method called reverse dot hybridization has been worked out by Saiki and colleagues.[54] In this method, mutation-specific oligonucleotides are separately immobilized onto a nylon membrane and a PCR product from genomic DNA of the patient is hybridized to the filter. The primers used in the PCR amplification are biotinylated so that hybridization of the PCR product to an oligonucleotide specific for a mutation is detected by rapid analysis for the presence of biotin in the oligonucleotide dots. This method should be useful for rapid detection of any known point mutation in genomic DNA.

PROBLEMS IN GENETIC COUNSELING

Over the past year as laboratory expertise has increased, genetic counselors have become more aware of potential risks to couples for bearing a child with one or another of the thalassemia syndromes. Couples of Mediterranean, Asian Indian, Chinese, Southeast Asian, Middle Eastern, and black backgrounds are often screened

for thalassemia trait by measuring their red blood cell indices and hemoglobin A_2 levels. For the vast majority of couples the test results provide clear answers; they are either at risk for being α- or β-thalassemia carriers or they are not at risk.

A problem occurs when one member is clearly a carrier and the second has both a borderline MCV and a borderline Hb A_2 concentration, e.g., MCV of 80 fl and Hb A_2 of 3.3%. This couple may well be at risk for β-thalassemia, since silent carrier alleles exist in Asian Indians and Mediterraneans. Indeed, a child with a silent carrier allele paired with a β^0 allele, e.g., a frameshift mutation, can have transfusion-dependent β-thalassemia major. In addition, mild β-thalassemia alleles can be associated with normal red blood cell indices and mildly elevated Hb A_2 concentrations. Therefore, in the situation described above we first obtain hematologic data on the parents of the possible carrier. If one of the parents also has "possible carrier" values, we opt to sequence the β-globin genes of the "suspicious" member of the couple. We believe that in such a couple a risk to the fetus of β-thalassemia exists and prenatal diagnosis should be undertaken.

Difficulty in detecting the 1–2% of β-thalassemia carriers who have very mild alleles poses a major problem for physicians and geneticists. Hematologic screening tests are accurate over 95% of the time, but they may err occasionally. Luckily the potential fallibility of screening tests is an issue only when the spouse is clearly a carrier by these tests. The problem then exists because a child who carries a β^0-thalassemia allele and a silent carrier allele may result, and such a child may have severe symptoms. The only present recourse for eliminating these rare inaccuracies of genetic testing is to carry out mutation analysis on all marginal individuals whose spouse is clearly a carrier. This type of study would eliminate all known causes of β-thalassemia in the ethnic group of the couple and cut the risk to the couple of an affected child to essentially zero.

EFFECTS OF SCREENING AND PRENATAL DIAGNOSIS IN NORTH AMERICA

In North America it has been difficult to estimate the effect of β-thalassemia screening and prenatal diagnosis. Clinics in major cities of the United States and Canada have seen a reduction in new patient referrals of between 50 and 80%. As genetic counseling centers in North America become more and more aware that the distribution of β-thalassemia extends beyond Mediterranean peoples to various Asians, Middle Easterners and blacks, more individuals in high-risk groups are screened and our ability to prevent β-thalassemia increases. The goal for North America should be the same successful prevention of the disease as now pertains in Greece and Italy. In the next 10 years, new patients entering β-thalassemia clinics in the United States and Canada should decrease to less than 10% of the number referred in 1975.

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The α-Thalassemias

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INTRODUCTION

The α-thalassemias are common genetic disorders that result from reduced synthesis of the α-globin chains of hemoglobin (Hb) (reviewed in Ref. 1). Most affected individuals originate from tropical and subtropical regions, where the high frequency is thought to have resulted from a selective advantage afforded to carriers of α-thalassemia in the presence of endemic *falciparum* malaria.[2] The clinically severe forms of α-thalassemia (Hb H disease and the Hb Bart's hydrops fetalis syndrome) are particularly common in Southeast Asia, where it has been estimated that up to 17,000 severely affected individuals are born each year.[3] Analysis of the determinants of α-thalassemia is directed towards providing a rational basis for genetic counseling and prenatal diagnosis of these disorders. In addition, much of the progress in our understanding of the mechanisms underlying globin gene expression has been derived from the analysis of these naturally occurring mutations.

THE NORMAL STRUCTURE AND EXPRESSION OF THE α-GLOBIN GENE COMPLEX

The α-globin cluster lies near the tip of chromosome 16, within band p13.3. It includes the duplicated α genes (α2 and α1), an embryonic α-like gene (ζ2), three pseudogenes (ψζ1, ψα2, ψα1), and a gene of undetermined function (θ1) arranged in the order 5′-ζ2-ψζ1-ψα2-ψα1-α2-α1-θ1-3′.[4-8] A pseudogene for the *Ro* family (ψρ) of small cytoplasmic RNAs has been identified downstream of the α1 gene,[9] and a truncated, processed copy of the θ1 gene family (ψθ2) has been found on chromosome 22.[10]

A striking feature of this region is that it contains several tandemly repeated segments of DNA (minisatellites). They were first identified as hypervariable regions (HVRs) located at the 3′ end of the complex (α-globin 3′HVR), between the ζ2 and ψζ1 genes (interzeta HVR), and within the introns (IVS-1 and IVS-2) of the ζ-like genes (ζ intron HVRs).[5,11-13] We have also identified other HVRs located in the 5′ flanking region of the cluster; a particularly informative polymorphic locus lies 70 kb upstream of the ζ2 gene (called the α-globin 5′HVR).[14] All of these hypervariable regions have been of great value in the genetic analysis of the α-globin cluster and of the 16p13.3 region in general. The structural features of the α-globin cluster are summarized in FIGURE 1.

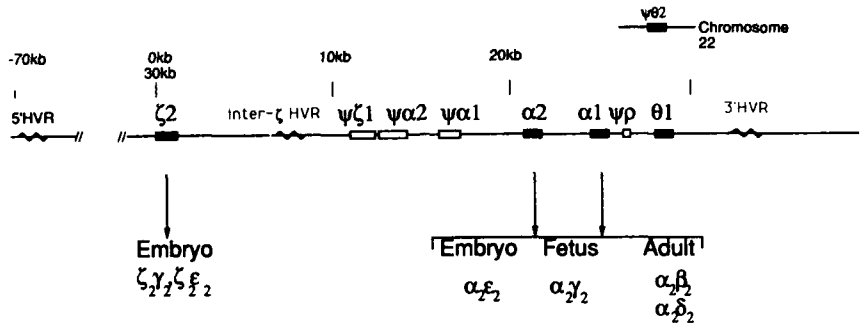

FIGURE 1. The structure and expression of the human α-globin gene cluster as described in the text. (**Upper line**) The positions of genes (*solid boxes*), pseudogenes (*open boxes*), and polymorphic hypervariable regions (HVR: *zig-zag lines*) are indicated. Note that the hypervariable regions in the introns of the ζ-like genes are not shown. (**Lower line**) The types of globin chains and hemoglobins synthesized at various stages of development are shown.

Both α and ζ genes are expressed in the primitive erythroblasts in the yolk sac (up to 6–7 weeks of gestation), although ζ-globin synthesis predominates during this period; definitive line erythroblasts almost exclusively synthesize α-globin (from 6 weeks onwards).[15] The expression of the α2 gene predominates over the α1 gene by approximately 3:1 throughout development.[16] Using more sensitive assays, low levels of ζ-globin expression can be detected throughout fetal life[17,18] and in up to 80% of cord bloods.[19] Similarly, low levels of θ mRNA can be detected at all stages of development.[18] None of these genes is expressed in non-erythroid tissues.

COMMON NATURALLY OCCURRING MUTATIONS THAT CAUSE α-THALASSEMIA

α-Thalassemia most commonly results from deletions of various segments of DNA from the α cluster. Deletions removing one of the duplicated α genes ($-\alpha$) are common throughout the "thalassemia belt." Deletions involving both α genes ($--$) are most commonly seen in Southeast Asia and the Mediterranean basin, where Hb H disease ($--/-\alpha$) and the Hb Bart's hydrops fetalis syndrome ($--/--$) occur. All such previously described deletions are summarized in FIGURE 2. It should be noted that the $-\alpha^{3.7}$ determinant has been classified into three subtypes depending on the position of the recombination event that has given rise to the α-gene deletion ($-\alpha^{3.7}$ type I, II, and III).[20] Small elevations in the level of ζ mRNA and ζ-globin chains are detectable in some but not all of these deletions (see Ref. 19 for further discussion).

Less commonly, α-thalassemia is the result of a single-base or an oligonucleotide mutation affecting either the α2- ($\alpha^T\alpha$) or α1- ($\alpha\alpha^T$) globin gene, so-called non-deletional α-thalassemia. Mutations of the dominant α2 gene produce a more severe phenotype than those affecting the α1 gene (reviewed in Ref. 1). Some cases of Hb H disease result from the interaction of non-deletional and deletional types of α-thalassemia (usually $--/\alpha^T\alpha$). Some homozygotes for non-deletional forms of α-thalassemia also have Hb H disease ($\alpha^T\alpha/\alpha^T\alpha$).[21,22] The currently described types of non-deletional α-thalassemia are summarized in TABLE 1.

DELETIONS ASSOCIATED WITH α-THALASSEMIA AND MENTAL RETARDATION

Occasionally deletions such as those summarized in FIGURE 2 may fortuitously occur in a patient with a mental handicap. However, we have recently described eight patients who have α-thalassemia that cannot be accounted for by the Mendelian inheritance of such determinants.[23] These patients have a mild-to-moderate mental handicap and a variety of dysmorphic features. Initial analysis of the α-gene cluster showed that the α-thalassemia resulted from a failure of these patients to inherit an α-globin allele from one parent. Further analysis has shown that all of these patients have large deletions removing the entire α-globin complex, including more than 1 megabase of DNA from the terminal band 16p13.3. In four cases the deletion results from unbalanced chromosome translocation, and, therefore, aneuploidy of a second chromosome is also present.[23] In the other four patients the mechanism responsible for the deletion has not been established, but it is possible that they are simply monosomic for part of the terminal band 16p13.3. At present, it is unclear whether there is an important locus in this region that might be responsible for the associated mental retardation. More cases of α-thalassemia due to pure monosomy for a portion of 16p13.3 will have to be analyzed before this hypothesis can be fully tested.

It should be noted that a second group of five patients with α-thalassemia and mental retardation has also been described.[24] All of these patients are profoundly mentally retarded individuals of north European origin, with an atypical form of Hb H disease. It is now clear that this syndrome, in which the α genes remain intact (so-called α-thalassemia/mental retardation: non-deletion), is quite different from the deletion form described above (see Refs. 23 and 24 for further discussion).

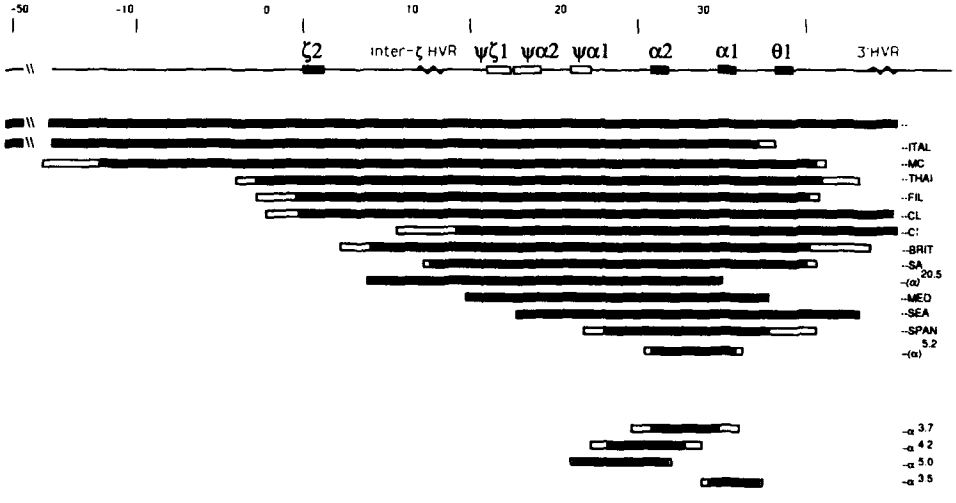

FIGURE 2. Summary of the deletions that give rise to α-thalassemia. (**Upper line**) The α-globin complex; genes are represented by *solid boxes*, pseudogenes by *open boxes*, and hypervariable regions (HVR) by *zig-zag lines*. (**Lower lines**) The extent of each deletion is represented as a *solid box*, and the uncertainty of the breakpoints is indicated by *open boxes*. These deletions and the primary references for them are summarized in detail in Ref. 1.

TABLE 1. Non-Deletion Mutants That Cause α-Thalassemia[a]

Mutant Class	Affected Gene	Affected Sequence	Mutation	Geographical Distribution
RNA processing	α2	IVS-1 donor site	GAGGTGAGG→GAGG-----	Mediterranean
	α2[b]	Poly(A) signal	AATAAA→AATAAG	Middle East, Mediterranean
RNA translation[c]	α2	Initiation codon	CCACCATGG→CCACCACGG	Mediterranean
	α1	Initiation codon	CCACCATGG→CCACCGTGG	Mediterranean
	−α	Initiation codon	CCACCATGG→CCACCGTGG	Black
	$-\alpha^{3.7\,kb}$	Initiation codon	CCACCATGG→CC--CATGG	North African, Mediterranean
	α2	Exon III	α116: GAC→UAG	Black
	α2	Termination codon	α142: TAA→CAA	Southeast Asian
	α2	Termination codon	α142: TAA→AAA	Mediterranean
	α2	Termination codon	α142: TAA→TCA	Indian
	α2	Termination codon	α142: TAA→GAA	Black
	−α	Exon I	α30/31: GAGAGG→GAG--G	Black
Post-translational instability	α2	Exon III	α125: Leu→Pro	Southeast Asian
	α2	Exon III	α109: Leu→Arg	Southeast Asian
	α	Exon III	α110: Ala→Asp	Middle Eastern
	−α	Exon I	α14: Trp→Arg	Black
Uncharacterized	α	Unknown	Not determined	Black
	α	Unknown	Not determined[d]	Greek

[a]Full details and references are summarized in Ref. 1.

[b]This mutation has been found in both α2-like genes on an $\alpha\alpha\alpha^{3.7}$ chromosome present in Saudi Arabian individuals.

[c]The elongated α chains associated with Hb Wayne, which results from a frameshift (deletion of either C at α138 or A at α139 of the α2-globin gene), and with Hb Grady, which results from a crossover in phase (with insertion of three residues at α118), are not known to be associated with α-thalassemia, although the critical interactions that would clearly reveal this have not been described.

[d]Interaction of this mutant with α^0-thalassemia determinants to produce the Hb Bart's hydrops fetalis syndrome suggests that both α-globin genes may be affected.

α-THALASSEMIA ASSOCIATED WITH A LARGE (62-kb) DELETION UPSTREAM OF THE α-GLOBIN CLUSTER

For most forms of α-thalassemia, detailed molecular analysis has readily explained the mechanism by which α-globin synthesis is reduced or abolished. However, we have previously described an English individual (RA) with α-thalassemia in which the molecular basis could not be so readily understood.[25] DNA analysis demonstrated a large (62-kb) deletion affecting one chromosome denoted $(\alpha\alpha)^{RA}$, spanning from between the ζ2 and ψζ1 genes to a position 52 kb upstream of the ζ2 mRNA cap site (FIG. 3). We have now demonstrated that the $(\alpha\alpha)^{RA}$ chromosome accounts for the α-thalassemia phenotype in this family.[26] Although this suggests even more strongly that the deletion is primarily responsible for the α-thalassemia, it is clearly impossible to rule out with absolute certainty the presence of a second inactivating mutation on this chromosome since not all of the *cis*-acting sequences required for α-globin gene expression have yet been identified.

Both α genes downstream of this deletion in the $(\alpha\alpha)^{RA}$ chromosome remain intact. However, no mutations were found within the transcription units of the α1 and α2 genes from the $(\alpha\alpha)^{RA}$ chromosome. Thus, the most plausible explanation is that the deletion is primarily responsible for down-regulating the expression of the

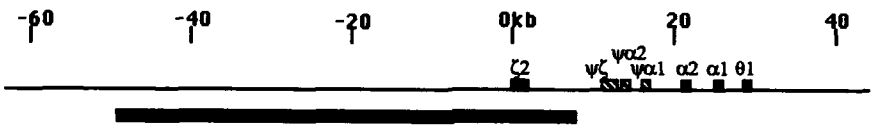

FIGURE 3. The extent of the deletion in the $(\alpha\alpha)^{RA}$ chromosome described in the text. (**Upper line**) The α-globin complex, as described in the legend to FIGURE 1. (**Lower line**) The extent of the $(\alpha\alpha)^{RA}$ deletion is indicated by a *solid box*. This deletion is described in detail in Refs. 25 and 26.

α-globin genes. This could be due to the juxtaposition close to the α-globin genes of a negative element from beyond coordinate −52. Alternatively, the large, 62-kb deletion could perturb the higher-order chromatin structure around the α-globin complex in a relatively non-specific manner, although other large insertions (+10 kb) and deletions (−10 kb) within the α complex appear not to alter the expression of the α genes in a significant way (reviewed in Ref. 1).

A third possible mechanism by which the deletion in the $(\alpha\alpha)^{RA}$ chromosome could inactivate the α genes is by the removal of a specific positive regulatory element that is essential for their expression. It has been demonstrated that such positive regulatory sequences exist 50–65 kb upstream of the human β-globin genes.[27] Furthermore, three different deletions that each remove these sequences severely down-regulate β-globin gene expression.[28–30] The positive regulatory sequences in the β-globin cluster [referred to as the β-dominant control region (β-DCR) or β-locus activating region (β-LAR)] confer high-level and position-independent expression on the β-like genes when constructs containing both the β-DCR and the gene(s) are transfected and integrated into the genome of MEL cells or transgenic mice.[27,31–33] Recently, it has also been shown that the β-DCR can exert a similar effect on the α-globin genes in these experimental systems.[34,35] Therefore, the deletion in the $(\alpha\alpha)^{RA}$ chromosome suggests the possibility of similar positive regulatory sequences upstream of the human α-globin cluster. Using constructs in which the segments of

DNA that are deleted from the $(\alpha\alpha)^{RA}$ chromosome are linked to an α-globin gene, we have shown that positive regulatory sequences do exist within this region.[36] This region imparts high-level expression to the α genes in both MEL cells and transgenic mice.

CONCLUSIONS

A large number of naturally occurring mutants of the α-globin cluster have been characterized, but further cases, particularly of the non-deletion types of α-thalassemia, will have to be studied in order to provide a comprehensive survey of such mutants. Mutants such as the $(\alpha\alpha)^{RA}$ determinant continue to provide valuable guides to regions of the α complex that are important in the control of gene expression. We now predict that deletions of the α-globin positive regulatory region will provide another class of determinants of α-thalassemia.

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The Use of Direct Gene Analysis to Define β-Thalassemia[a]

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The diagnosis of β-thalassemia has progressed from phenotype diagnosis in the 1960s and 1970s to genotype diagnosis in the 1980s. By means of hemoglobin electrophoresis and measurement of globin chain synthesis, β-thalassemias were first classified into heterozygous or homozygous forms of the β^0 or β^+ type.[1] When DNA analysis was first introduced, restriction enzyme polymorphism was initially used for linkage analysis of thalassemia chromosomes.[2] With the delineation of precise mutations that cause thalassemia, direct detection of mutations was possible either by restriction enzyme analysis[3] or by oligonucleotide probe hybridization.[4] The advent of the polymerase chain reaction (PCR),[5] in which a given segment of DNA can be amplified many millionfold *in vitro,* has given the detection of thalassemia mutations a new dimension. At present, a number of alternate methods for the clinical detection of β-thalassemia are available, as summarized in TABLE 1. Following amplification of the β-globin gene sequences, mutations can be detected by restriction enzyme digestion in those cases where the mutation either creates or abolishes a restriction site[6] or by hybridization with allele-specific oligonucleotide probes.[7] Alternatively, allele-specific oligonucleotide primers can be used to prime differentially normal or mutant sequences.[8] Rapid methods are also available to detect unknown mutations. They include denaturing gradient gel electrophoresis,[9] single-strand conformation polymorphism electrophoresis,[10] and chemical[11] or enzymatic cleavage.[12] In this paper, we will discuss some of the methods we have used in our laboratory to detect mutations in β-thalassemia.

METHODS FOR KNOWN MUTATIONS

Restriction Enzyme Digestion

The prototypic example of detection by restriction enzyme digestion is the assay for the sickle cell mutation. Here, the A→T mutation abolishes the recognition sites of several isoschisomers that cleave at the sequence CTNAG.[6] Amplified DNA from

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TABLE 1. Direct Detection of Thalassemia Mutations with PCR

Known Mutations
Restriction enzyme digestion
Allele-specific oligonucleotide probe hybridization
Allele-specific oligonucleotide primer amplification
Known and Unknown Mutations
Denaturing gradient gel electrophoresis
Single-strand conformation polymorphism electrophoresis
Chemical or enzymatic cleavage of mismatches

the normal chromosome is cleaved by this enzyme, while that from the sickle cell mutation is not (FIG. 1). Of the over eighty point mutations that have been defined to date in β-thalassemia, approximately one-third could be detected in this manner because they either create a new restriction site or abolish an existing one.[13]

Allele-specific Oligonucleotide Probe Hybridization

For hybridization with allele-specific oligonucleotide probes, the PCR-amplified DNA is dotted and immobilized on a nylon or nitrocellulose filter and hybridized

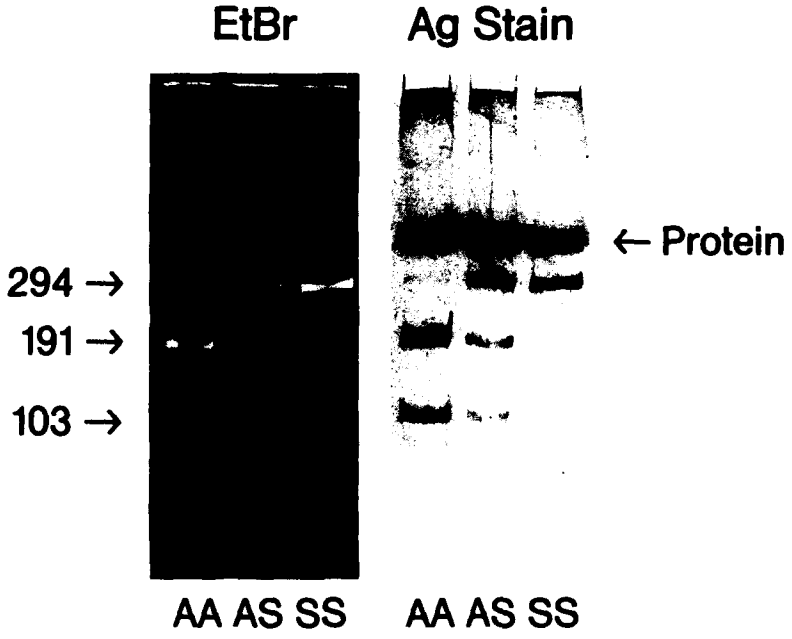


FIGURE 1. Detection of the sickle cell mutation by restriction enzyme digestion of amplified DNA. A 294-bp segment of DNA was amplified and cleaved with the enzyme OXAN-1, which recognizes the sequence CCTNAGG. The DNA from the normal chromosome (A) was cleaved into two segments of 191 and 103 bp, while the DNA from the sickle chromosome (S) was not. (**Left panel**) Ethidium bromide stain, (**right panel**) silver stain. (From Chehab *et al.*[6] Reprinted by permission from *Nature*. Copyright 1987 by Macmillan Magazines Ltd.)

with a pair of oligonucleotide probes corresponding to either the normal or the mutant sequence.[7] The probes can be labeled with ^{32}P, ^{35}S, or horseradish peroxidase (FIG. 2). One pair of probes is used for each mutation. This method is presently most widely employed for the detection of point mutations in β-thalassemia and other genetic diseases.

A modification of this method utilizes the principles of reverse oligonucleotide hybridization.[14] Here, the oligonucleotide probes that are homologous to the normal or to the mutant alleles are immobilized on a filter and hybridized with the amplified test DNA, which has been labeled with biotinylated nucleotides. The hybridization can be detected by different colorimetric reactions on the filter, such as that of horseradish peroxidase. The advantage of this approach is that all the mutations to be investigated can be immobilized on one filter, and only a single hybridization with the test DNA is necessary.

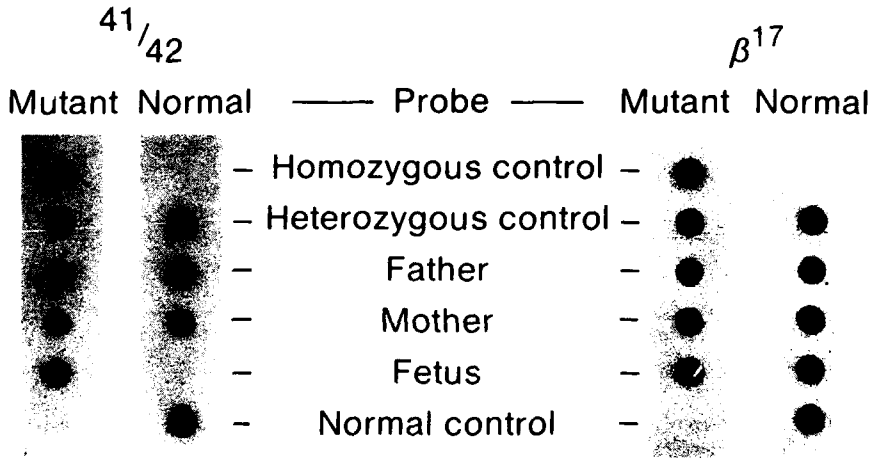


FIGURE 2. Dot blot hybridization of PCR-amplified DNA. (**Left panel**) Fetus homozygous for a 4-bp deletion at codons 41 and 42, (**right panel**) fetus heterozygous for the β^{17} nonsense mutation. Allele-specific oligonucleotide probes for the mutant (**left lanes of each panel**) or normal (**right lanes**) sequences were used for the hybridization, as indicated. (From Cai *et al.*[19] Reprinted with permission from *Blood*.)

Allele-specific Oligonucleotide Primer Amplification

In this method, three primers are used for the PCR reaction, two being complementary to the region of the mutation and identical to each other except for the sequence corresponding to the mutation. The third primer is used to extend the opposite strand. The primers which are completely homologous will preferentially extend in the PCR reaction. Thus, with the normal chromosome, only the primers corresponding to the normal sequence will extend, while with the abnormal chromosome, only the mutant DNA primer will extend. Two separate reactions can be used for each test sample, one with the normal and the other with the mutant primer (FIG. 3). Alternatively, the normal and mutant products can be identified by labeling the primers radioactively[15] or with fluorescent dyes of different colors,[8] and competitive PCR can then be performed in a single reaction. In the latter example, normal, heterozygous and homozygous states can be detected by observing the color of the

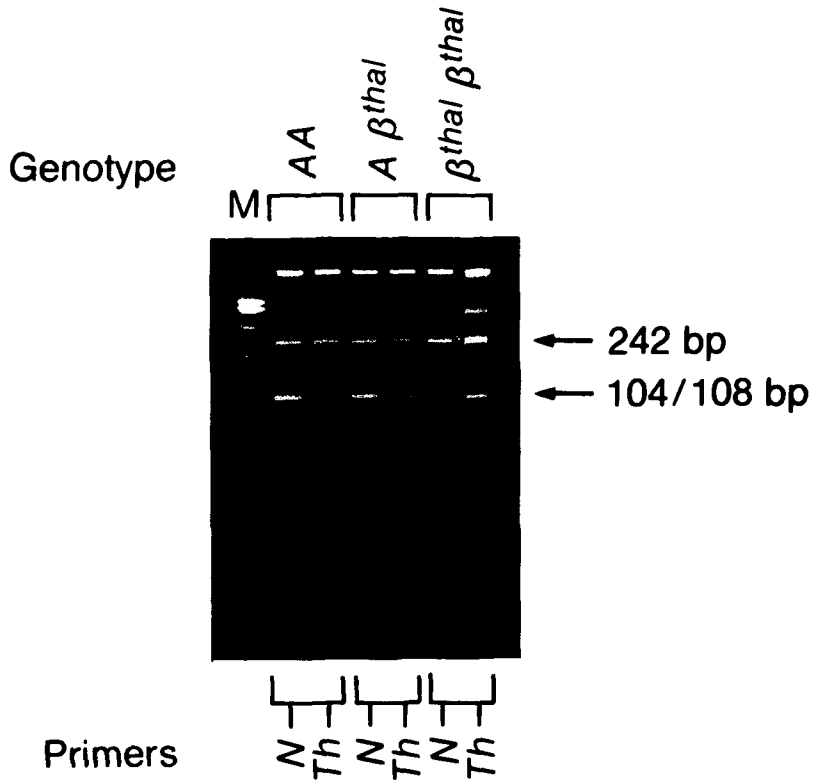


FIGURE 3. Detection of the normal and the mutant alleles by allele-specific oligonucleotide primers. The mutation was a β-thalassemia due to a 4-bp (CTTT) deletion at codons 41/42. In each reaction, either the normal (N) or the mutant (Th) primer was used with the primer of the opposite strand. The amplified product was 108 (normal) or 104 (mutant) bp long. A 242-bp segment of another region of the β-globin gene was co-amplified as an internal control. M, marker; AA, normal; A β^{thal} and β^{thal} β^{thal}, heterozygous and homozygous, respectively, for the 4-bp deletion. The unlabeled bands are due to nonspecific priming.

PCR product (FIG. 4). A potential advantage of this method is that it is adaptable to laser scanning and automation.

METHODS FOR KNOWN AND UNKNOWN MUTATIONS

The above three methods are used when the mutations to be detected have been defined. However, uncharacterized mutations may occasionally be encountered. With the following methods, one can detect known mutations and also be alerted to the presence of new, undefined mutations.

Denaturing Gradient Gel Electrophoresis

In this method, the β-globin gene is divided into segments of about 150 to 400 bp for amplification.[16] One of the PCR primers has a GC tail of approximately 40

nucleotides, thereby adding a GC clamp to the PCR products. The amplified DNA is separated by electrophoresis on a denaturing gradient gel. As each DNA segment reaches the position on the gradient at which it denatures, its migration in the gel is retarded. Thus, each segment has its own characteristic mobility, which can be altered by a point mutation within it. An example is shown in FIGURE 5. The three samples are heterozygous for the three TATA box mutations (the −28 A→G, the −29 A→G, and the −30 T→C). While a single band is seen in the normal sample, four bands are discernible in the heterozygous samples: a homoduplex of the normal fragment, a homoduplex of the mutant fragment, and two heteroduplexes of the normal and abnormal fragments. The homoduplexes of the three mutant alleles migrated differently from that of the normal, and the homoduplex of the −28 mutation differs from those of the −29 and −30 mutations. Although the homoduplexes of the −29 and −30 mutations have the same mobilities, their respective heteroduplexes are quite different. Hence, one can define known mutations by comparing the mobilities of the homoduplexes and heteroduplexes to those of known standards, and one can detect new ones by observing new patterns.

Single-Strand Conformation Polymorphism Electrophoresis

In this technique, PCR-amplified DNA fragments are denatured in alkaline solution and run on a neutral polyacrylamide gel. Point mutations affect the conformation of the single-strand DNAs and alter their mobility.[10] Thus, as in

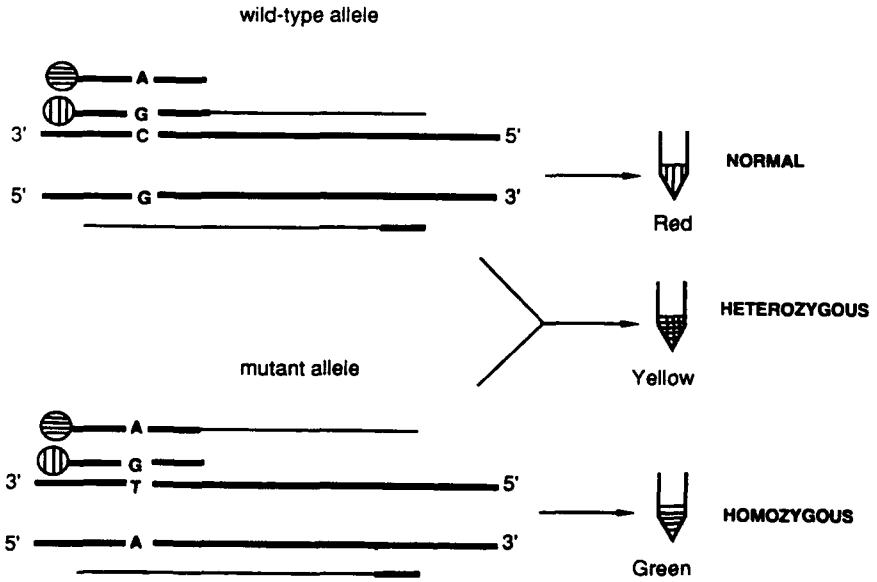
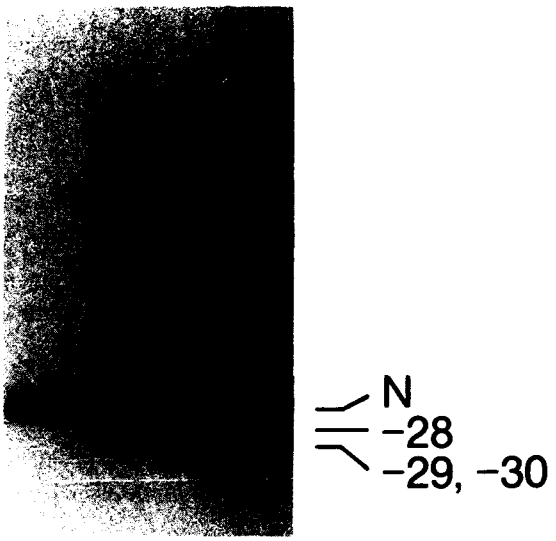


FIGURE 4. Principle of PCR with fluorescent dye–labeled allele-specific primers. The primer complementary to the normal allele was labeled with rhodamine (*circles with vertical bars*), and the primer complementary to the mutant allele with fluorescein (*circles with horizontal bars*). The PCR products of the normal alleles fluoresce red, the homozygously affected green, and the heterozygous yellow. (From Chehab & Kan.[8] Reprinted with permission from the *Proceedings of the National Academy of Sciences of the United States of America.*)



Normal	+/+	+	+	+
−28		+		
−29			+	
−30				+

FIGURE 5. Denaturing gradient gel electrophoresis. The four samples are one normal (**left lane**) and three heterozygous for one of the mutations at the TATA box. The position of the normal (N) and mutant (−28, −29, −30) homoduplexes are indicated. The heteroduplexes migrated more slowly and differed from each other in the three mutant DNAs. (From Cai & Kan.[16] Reprinted with permission from the *Journal of Clinical Investigation.*)

denaturing gradient gel electrophoresis, mutations can also be detected by their difference in mobilities.

Chemical and Enzymatic Cleavage

Both of these methods use a normal β-globin gene probe for hybridization to the test DNA, and they detect mutations by chemical or enzymatic cleavage at the site of the mismatched sequence.[11,12] Hence, not only can new mutations be detected, but also the site of these mutations can be located. These methods, however, are not usually suitable for diagnostic purposes, as cleavage is not always complete and the heterozygous and homozygous state cannot always be distinguished with certainty.

CONCLUSION

The present strategy for diagnosis of β-thalassemia is detection of the mutations. Although over 80 point mutations have been described world-wide to date, each geographical location has its own characteristic distributions of mutations, which have been carefully mapped out by many groups of investigators. It has been estimated that in most ethnic groups, the use of the appropriate four to five different probes could cover over 90% of the mutations in each location.[17] As a primary test, restriction enzyme digestion, oligonucleotide probe hybridization, or oligonucleotide primers could be used. They could be supplemented by denaturing gradient gel electrophoresis or single-strand conformation polymorphism electrophoresis, by which the rarer mutations can also be detected. The introduction of PCR allows the application of non-radioactive methods and makes DNA diagnosis of β-thalassemia feasible in many areas of the world. A positive impact of these methods on the control of thalassemia is already seen in countries where β-thalassemia is an important genetic problem.[18]

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β-Thalassemia in Thailand[a]

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INTRODUCTION

β-Thalassemia and hemoglobin (Hb) E are prevalent in Thailand. The frequencies of β-thalassemia vary from 3% in central Thailand to 9% in the north. The frequency of Hb E is 13% on the average; and its distribution is heterogeneous, attaining 50–60% at the junction with Laos and Cambodia.[1-4] β^0-thalassemia is much more common than β^+-thalassemia. Homozygous β^0-thalassemia and double heterozygosity between β^0- and β^+-thalassemia as a rule result in severe Cooley's anemia disease. While the Hb E homozygote is asymptomatic, double heterozygosity between β^0-thalassemia and Hb E (β^0-thalassemia/Hb E) can be a very severe disease; a spectrum of severity with hemoglobin levels ranging from 3 to 13 g/dl has been observed.[5] β^0-Thalassemia/Hb E is the most common β-thalassemic disease in Southeast Asia.

At present there are more than 70 different mutations resulting in β-thalassemia, most of which are single base changes in and around the β-globin gene. It has been found that, in general, β-thalassemia mutations are relatively population specific, i.e., each ethnic group has its own set of common mutants. The purpose of this paper is to review the nature of the molecular defects of β-thalassemia in Thailand and also to compare the patterns with those in neighboring countries.

MOLECULAR DEFECTS OF β-THALASSEMIA IN THAILAND

The data for this review are derived from four studies on a total of 319 β-thalassemic genes. Background information for the four studies is presented in TABLE 1. There can be two main biases in the subjects examined. First is the different ethnic admixture of the people in various localities of the country. Second is the bias

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TABLE 1. Background Information for Studies on β-Thalassemia in Thailand

Authors	No. of Chromosomes	Locality	Disease and No. of Cases[a]
Petmitr *et al.*[6]	25	Central	β-Thal/Hb E (25)
Fucharoen *et al.*[7]	71	Central, south, and northeast	β-Thal/Hb E and homozygous β-thal
Thein *et al.*[8]	119	Central	β-Thal/Hb E (81) and homozygous β-thal (19)
Winichagon *et al.* (this report)	104	Central	β-Thal/Hb E (24) and homozygous β-thal (40)

[a]β-Thal, β-thalassemia. Number of cases for each disease is shown in parentheses.

occurring from different β-thalassemia diseases. Among the β-thalassemia/Hb E patients there will be more with the molecular defects leading to β^0-thalassemia, while among β-thalassemia/β-thalassemia patients there will be increased proportions of the defects leading to β^+-thalassemia. This is because β^+-thalassemia/Hb E is mild and mostly not seen in the hospitals.

TABLE 2 summarizes the molecular defects of β-thalassemia found in the four studies. Eleven mutations have been detected. Among these, four are common: a 4-bp deletion leading to frameshift at codons 41/42 (43% frequency), a nonsense mutation at codon 17 (14%), a C→T substitution at position 654 of IVS-2 (10%), and an A→G transition at position −28 of the ATA box (6%). A G→C mutation at IVS-1, nucleotide (nt) 5, occurs in 5% of the patients, but this is more frequent in the south, where spillover from Malaysia is expected.[7] A higher proportion of the mutation A→G at codon 19, which leads to the production of the variant β^{Malay} chain, is also observed in the patients originating from southern Thailand. In addition, a 619-bp deletion at the 3′ end of the β-globin gene is detected in Indian immigrants suffering from homozygous β-thalassemia. Two novel mutations are found in the

TABLE 2. β-Thalassemia Mutations in Thailand Characterized from Patients Affected with Homozygous β-Thalassemia or β-Thalassemia/Hb E Disease

Mutation	Study: Petmitr *et al.*[6]	Fucharoen *et al.*[7]	Thein *et al.*[8]	This Report	Total	Frequency
β^0-Thalassemia						
Codons 41/42	14	31	59	32	136	42.6%
Codon 17	3	14	12	17	46	14.4%
IVS-2 nt 654	1	9	13	8	31	9.7%
Codon 35	1	1	3	1	6	1.9%
Codons 71/72	0	2	1	2	5	1.6%
619-bp deletion	0	0	0	5	5	1.6%
IVS-1 nt 1	0	1	2	1	4	1.3%
β^+-Thalassemia						
ATA nt −28 (A→G)	0	2	12	6	20	6.3%
IVS-1 nt 5	1	8	6	1	16	5.0%
Codon 19	0	3	2	4	9	2.8%
nt −86 (C→G)	ND[a]	0	1	ND[a]	1	0.3%
Unknown	5	0	8	27	40	12.5%
No. of chromosomes	25	71	119	104	319	

[a]ND, not determined in this study.

Thai patients. First, a C→A ochre mutation in codon 35, leading to β^0-thalassemia,[9] occurs at a frequency of 2%. Second, a G→C mutation at position −86 was found in a homozygous β-thalassemia patient studied by Thein *et al.*[8] The molecular basis of β-thalassemia is still unknown in 13% of the patients reported in these studies. It is suspected that there may exist in these patients mutations which are unique and hitherto have not been reported.

TABLE 3 and FIGURE 1 summarize the patterns of common β-thalassemia mutations in Thailand and neighboring countries. Several patterns are observed, as detailed in the following paragraphs.

Chinese/Thai Pattern

The Thai and the Chinese have a similar pattern of β-thalassemia mutations (frameshift 41/42, amber 17, IVS-2 nt 654, ATA nt −28).[6-12] It is characterized by the preponderance of a frameshift mutation at codons 41/42, which constitutes almost 50% of all the defects, followed in frequency by an amber mutation at codon 17, and mutations at IVS-2 nt 654 and nt −28 of the ATA box. Frameshift 41/42 appears to have the highest proportions in southern China and Thailand, but it is not limited to this region. The other three mutations are confined to Chinese/Thai. The Thai have extensive Chinese admixture; whether the "pure" Thai have a pattern of β-thalassemia mutations different from the Chinese/Thai pattern should be investigated.

Asian Indian Pattern

This pattern (IVS-1 nt 5 Indian type, 619-bp deletion, frameshift 8/9, IVS-1 nt 1) is characterized by equal proportions of IVS-1 nt 5, a 619-bp deletion and a frameshift at codons 8/9, followed in frequency by IVS-1 nt 1.[15] IVS-1 nt 5 in the Indians differs in haplotype and framework from that in the Melanesians. Among Indians, IVS-1 nt 5 is associated with + − − − − − + and − + + − + − +, while among Melanesians it is with + − − − − + +.[16] The 619-bp deletion is characteristic of Asian Indians, and frameshift 8/9 is not detected among other Asians.

Melanesian Pattern

This pattern (IVS-1 nt 5 Melanesian type) is characterized by the almost sole presence of IVS-1 nt 5 associated with the + − − − − + + haplotype and framework, or Melanesian type.[16] The presence in high proportion of IVS-1 nt 5 Melanesian type among the Malaysians is compatible with the Malayo-Polynesian ethnic background of the people. It is predicted that IVS-1 nt 5 Melanesian type is a common β-thalassemia mutation among other Malayo-Polynesian ethnic groups, like the Indonesians and Filipinos.

Burmese Pattern

In this pattern (IVS-1 nt 5 Indian type, IVS-1 nt 1, frameshift 41/42), the preponderant β-thalassemia mutations are IVS-1 nt 5 Indian type, IVS-1 nt 1, and frameshift 41/42 Chinese type.[13] Conspicuously low proportions of an amber muta-

TABLE 3. Patterns of Common β-Thalassemia Mutations in Thailand and Neighboring Countries

β-Thalassemia Mutation	Thailand (%)	China[10-12]			Burma[13] (%)	Malaysia[14] (%)	India[15] (%)	Melanesia[16] (%)
		Guangdong (%)	Guangxi (%)	Sichuan (%)				
Codons 41/42	43	46	48	30	16	12	12	
Codon 17	14	10	29	30	6			
IVS-2 nt 654	10	19		11		7		
ATA nt −28	6	11	8	8	6			
Codons 71/72			9					
ATA nt −29				19				
IVS-1 nt 5					29	49	23[a]	87[b]
IVS-1 nt 1					25	7	14	
Codon 19						15		
619-bp deletion							22	
Codons 8/9							20	

[a]Indian type.
[b]Melanesian type.

tion at codon 17 and a mutation at nt −28 of the ATA box characteristic of the Chinese/Thai pattern are also detected.

Malaysian Pattern

The Malaysian pattern (IVS-1 nt 5 Melanesian and Indian types, codon 19, and IVS-2 nt 654)[14] reflects its people's ethnic backgrounds. The Malayo-Polynesian background is represented by IVS-1 nt 5 Melanesian type, the Indian by IVS-1 nt 5 Indian type, and the Chinese by IVS-2 nt 654 and frameshift 41/42. A mutation at codon 19 leading to Hb Malay is characteristic of the Malaysian pattern.

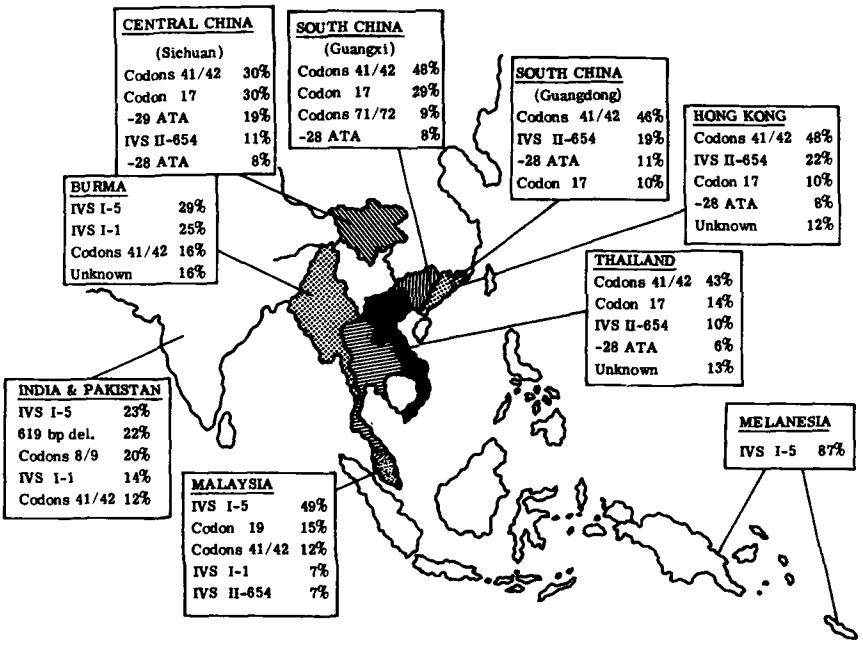

FIGURE 1. Summary of the common β-thalassemia mutations found in Thailand and neighboring countries (Refs. 6–8, 10–16, and this report).

DNA POLYMORPHISM ASSOCIATED WITH β-THALASSEMIA

Although several different 5′ haplotypes of DNA polymorphism have been observed in the chromosomes bearing β-globin genes, only three of them, i.e., +−−−−, −+−++, and −++−+ are common in all populations; and the −−−−+ haplotype is mainly found in Africans.[17] These haplotypes are non-randomly associated with β-globin gene frameworks designated as follows: framework 1, ++; framework 2, +−; and framework 3, −+. However, the +−−−− haplotype associated with framework 1 or 3 is often observed in the normal β-globin genes and in β-thalassemia genes. It has been noticed that, in a certain population, different

β-thalassemia mutations tend to be associated with particular haplotypes and frameworks.

TABLE 4 shows the association of DNA haplotypes and frameworks with β-thalassemia mutations in the Thai and other ethnics in neighboring countries. Only common β-thalassemia mutations will be discussed in the following paragraphs.

Frameshift Mutation at Codons 41/42

A frameshift mutation at codons 41/42 is common among the Asian populations; its focus is in southern China and Thailand. It has been found to be strongly associated with the 5′ haplotype +−−−− (TABLE 4). However, occurrence of this mutation on chromosomes with different sets of β-globin gene frameworks suggests multiple origins for this mutant. Two frameworks, 1 and 3, are detected in the Thai and Chinese but, as found in our present study, the Thai frameshift 41/42 is linked to framework 3 (−+) in slightly higher proportion (19/33 alleles), while the Chinese one is mainly linked with framework 1 (++: 29/37 alleles).[10,11] The Burmese mutation also has the framework of the Chinese type.[13] The Malaysian and Indian frameshift 41/42 mutations mostly occur on framework 2 (+−): 4/5 alleles in the Malaysian group and 9/12 alleles in the Indian. The remaining frameshift 41/42 mutations in these groups are on the β-gene with framework 1 (++).[14,15]

Mutations in Codon 17, IVS-2 nt 654, and ATA Box nt −28

These mutations are commonly found in the Thai, Chinese, Burmese and Malaysian groups (TABLE 3). Although more than one pattern of DNA polymorphism is observed, only one particular haplotype predominates in each mutation. The amber mutation at codon 17 is linked to +−−−− −+, IVS-2 nt 654 mostly to +−−−− ++, and the mutation at nt −28 of the ATA box to −++−+ −+ in the Thai and Chinese (TABLE 4).

G→C Mutation in IVS-1 nt 5

A G→C mutation in IVS-1 nt 5 has been found at a high frequency among the Melanesian, Malaysian, Burmese, and Asian Indian groups (TABLE 3). Although many different 5′ haplotypes are detected, only two β-gene frameworks, −+ and ++, each of which is present in a particular racial group, are observed (TABLE 4). This indicates a dual origin for the occurrence of this β-thalassemia mutant. Among Melanesians, IVS-1 nt 5 is mostly associated with the +−−−− ++ haplotype (Melanesian type),[16] while among the Indians it is associated with +−−−− −+ (Indian type).[13,15] The Burmese have the IVS-1 nt 5 defect of the Indian type. The presence of both types in the Malaysians reflects the admixture of these two racial groups in Malaysia.[14]

G→T Mutation in IVS-1 nt 1

This β-thalassemia mutant is common in Burmese and Indians and is found in a certain proportion of Malaysians (TABLE 3).[13-15] Only one pattern of DNA polymorphism, −+−++ ++, has been observed in the Asian Indians (TABLE 4). This

TABLE 4. Association of DNA Haplotypes and Frameworks with β-Thalassemia Mutations in the Thai and Other Ethnics in Neighboring Countries

β-Thalassemia Mutations	Haplotypes and Frameworks[a]					
	Thai[b]	Chinese (Guangdong)[10,11]	Burmese[13]	Malaysian[14]	Indian[15]	Melanesian[16]
Codons 41/42	+−−−− −+ +−−−− ++	+−−−− −+ +−−−− ++	+−−−− −+ +−−−− ++	+−−−− ++ +−−−− +−	+−−−− ++ +−−−− +−	None
Codon 17	+−−−− −+	+−−−− −+	+−−−− −+	Not done	None	None
IVS-2 nt 654	+−−−− ++	+−−−− ++ +−−−− −+	+−−−− ++	+−−−− ++ +−−−− −+	None	None
ATA nt −28	−++−+ −+ +−−−− −+	−++−+ −+	+−−−− −+	None	None	None
IVS-1 nt 5	Few	Few	+−−−− −+ −++−+ −+[c] −+−++ −+[c]	+−−−− −+ +−−−− ++	+−−−− −+ −++−+ −+[c]	+−−−− ++
IVS-1 nt 1	Few	None	+−−−− ++ −+−++ ++[c] −+−++ +−[c]	+−−−− ++	−+−++ ++	None
619-bp deletion	None	None	None	None	+−−−− −+	None
Codons 8/9	None	None	None	None	+−−−− ++ −+−++ ++[c] −++−+ ++[c]	None
Codon 19	Few	None	None	−++−+ +−	None	None

[a]Only predominant haplotypes and frameworks are shown. DNA haplotypes and frameworks are as reported by Antonarakis *et al.*[17]: *Hinc* II/ε, *Hind* III/^{G}γ, *Hind* III/^{A}γ, *Hinc* II/ψβ, *Hinc* II/3′ψβ, *Ava* II/β, and *Bam*H I/3′β.
[b]Data shown are from this report.
[c]These haplotypes and frameworks are found in lower frequencies.

−+−++ ++ is also predominant in the Burmese IVS-1 nt 1 (18/24 alleles), while two other haplotypes, −+−++ +− and +−−−− ++, are also detected at an equal proportion (3/24). In the Malaysian IVS-1 nt 1, only +−−−− ++ has been found.

619-bp Deletion and Frameshift Mutation at Codons 8/9 in Asian Indians and Mutation at Codon 19 in Malaysians

While the 619-bp deletion and frameshift 8/9 are characteristic of the Asian Indians,[15] the mutation at codon 19 (Hb Malay) is found only in Malaysians.[14] The single haplotypes, +−−−− −+, associated with the Indian deletion type, and −++−+ +−, associated with Hb Malay, indicate single origins for each of these mutations. The frameshift mutation at codons 8/9 also occurs with a single origin (framework 1: ++), although three different 5′ haplotype have been observed (TABLE 4).

DNA POLYMORPHISM ASSOCIATED WITH THE β^E GENE

Antonarakis *et al.,* in examination of 23 chromosomes bearing β^E genes from Cambodians, Laotians, and Thai, found that the β^E gene is situated in two β-gene frameworks, i.e., framework 3 (−+) and framework 2 (+−).[17] This suggests that there are two independent mutations for the common Southeast Asian Hb E. Hundrieser *et al.* found that Hb E in Cambodia is mainly associated with framework 3, while Hb E in the Thai, in the Vietnamese and among the Kachari in Assam, a Tibeto-Burman ethnic, is associated with framework 2.[19,20] Furthermore, Hundrieser *et al.* have found a gradient in the distribution of the two types of Hb E in Cambodia. In eastern Cambodia, framewrok 3 Hb E is almost singly present, while in western Cambodia bordering Thailand, framework 2 Hb E appears in a low frequency. Yenchitsomanus et al.[18] in our laboratory examined 22 samples of homozygous Hb E in Thailand. We have now examined 47 additional samples of Hb E from β-thalassemia/Hb E patients. These two studies also reveal that Hb E in Thailand is mainly associated with framework 2, although framework 3 Hb E is also detected in much lower frequency (TABLE 5).

Since framework 3 Hb E is concentrated in Cambodia, it may be called Cambodian Hb E. In Thailand, framework 2 Hb E is the main mutant; thus, framework 2 Hb E may be called Thai Hb E. The common Hb E so far reported in other Southeast Asian countries is the Thai Hb E. There is of course diffusion of Cambodian Hb E and Thai Hb E across the boundaries of countries.

At the junction of Laos, Thailand and Cambodia, the frequencies of Hb E are as high as 50–60%, and the area has been dubbed the "Hb E triangle." Originally it was thought that the Thai and the Laotians had acquired Hb E from admixture with the Cambodians. The existence of distinct Cambodian and Thai Hb E, as revealed by DNA polymorphism studies, indicates that this is not the case. It also suggests that selective pressure for Hb E at the "Hb E triangle" must have operated for both types of Hb E.

It is interesting to note that in Assam, in India, bordering Burma, Hb E has been found in spectacularly high frequencies in at least three different ethnic groups, i.e., the Thai Ahom, the Khasi, and the Kachari.[22] Hundrieser *et al.* have reported that Hb E among the Kachari, which is a Tibeto-Burman ethnic, is of the framework 2 (Thai) type.[20] The types of Hb E among the other two ethnics have not been reported. It can

TABLE 5. Distribution of β^E-Globin Gene Framework in the Thai and Other Ethnics[a]

Framework	Thai (%)				Cambodian[19] (%)	Vietnamese[20] (%)	Kachari in Assam[21] (%)
	Central[b]	Central[18]	North[19]	Northeast[19]			
2 (+ −)	85	86	100	97	35	77	100
3 (− +)	15	14	0	3	65	23	0
No. of chromosomes	*47*	*44*	*22*	*40*	*46*	*22*	*46*

[a]Data are summarized from five studies, in which the two restriction polymorphic sites were *Ava* II and *Bam*H I.
[b]Data shown are from this report.

be expected that Hb E in the Thai Ahom would be of the Thai type. The Khasi is Mon-Khmer in ethnic. It would thus be extremely interesting to know whether the Khasi has Cambodian Hb E or Thai Hb E. There are 64 Mon-Khmer dialects spoken by people who are scattered in different countries.[23,24] Besides the Mon and Cambodians, which represent the major groups, the Mon-Khmer–speaking people include several minorities, such as the Sagai in Malaysia, the Khamu, the Yambri, the Kha, the So, and the Suay. In all the Mon-Khmer–speaking ethnics that have been examined, Hb E is found in high frequencies. It has been reported that Hb E in the So living in northeastern Thailand is associated with framework 2 (Thai Hb E).[25] The Thai ethnics are scattered in eight countries in this area; the Laotians and the Thai belong to the same ethnic. Knowledge on the distribution of Cambodian Hb E and Thai Hb E in the Mon-Khmer ethnics and Thai ethnics will bring better understanding about the origin and migration of these people as well as the chronology of the occurrence of Hb E mutations.

SUMMARY

The nature of the genetic defects in β-thalassemia in Thailand was reviewed from four studies involving 319 β-thalassemia genes. Eleven mutations were identified. Among these four were common: a 4-bp deletion at codons 41/42 (43% frequency), an amber mutation at codon 17 (14%), a C→T substitution at IVS-2 nt 654 (10%), and a A→G substitution at ATA box nt −28 (6%). The pattern of common β-thalassemia genes among the Thai and the Chinese is similar.

In neighboring countries there are specific patterns of common β-thalassemia genes. The Indian pattern consists of IVS-1 nt 5, a 619-bp deletion, frameshift 8/9, and IVS-1 nt 1. The Melanesian pattern is characterized by the almost sole presence of IVS-1 nt 5. The IVS-1 nt 5 mutations of Indians and Melanesians are associated with different β gene frameworks. The Burmese pattern is a partial mixture of the Indian and the Chinese/Thai pattern. The Malaysian pattern reflects Chinese, Indian and Malayo-Polynesian ethnics.

DNA polymorphisms on chromosomes bearing the β^E gene differ considerably from those of the normal β and β-thalassemia genes. It has been suggested that the β^E mutation originated on the −+−++ haplotype. Two β-gene frameworks, 2 (+−) and 3 (−+), are linked to the β^E gene. While framework 2 Hb E (the Thai Hb E) predominates in the Thai and most ethnic groups in Southeast Asia, framework 3 Hb E (the Cambodian Hb E) is more frequent in the Cambodians.

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β-Thalassemia in Algeria

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INTRODUCTION

As in other Mediterranean countries, where malaria was endemic, thalassemias are frequent in Algeria. The epidemiology of α-thalassemia is only partially known, but studies performed in various population groups—blood donors, school children, tribes from the south of the country—have found a gene frequency for β-thalassemia ranging from 2.5 to 3 per 100. Given the present demography (30×10^6 inhabitants, 850,000 births per year), one should expect more than 100 new homozygous cases to be born every year. A similar estimate, 125 cases, has been calculated on the basis of the forms associated with hemoglobin variants.[1] Therapeutic possibilities are still limited, and life expectancy therefore varies in different locations. It nevertheless seems reasonable to assume the existence of living patients approaching one thousand in number, even though presently identified cases reach only one-half of this number.

Over several years we have been able to perform an extensive study, focused mostly on patients transfused in Algiers itself, but also involving cases from other parts of the country. This study has taken several different approaches:

1. Many patients have been carefully investigated before receiving any treatment, therefore providing data concerning the natural history of the disease and its phenotypic expression.
2. With the facility brought by the polymerase chain reaction, it was possible to establish a good estimation of the frequency of the various mutations, both common and rare. Novel mutations have been identified. This screening has presently involved the study of 173 chromosomes.
3. Together with the mutations themselves, the chromosome background on which they arose has been explored, initially with the classical haplotype data. The regions controling the expression of the γ genes have also been further explored, as was the direct environment of the β gene itself. From these sets of data, it is possible to propose hypotheses concerning both the phenotypic expression and the possibility of various genetic events.

NATURAL HISTORY OF β-THALASSEMIA IN ALGERIA

About 80 patients are regularly followed at the Transfusion Center in Algiers, but many more have been seen in recent years, due to improved communication with affected families. Within a relatively short time, a cohort of 50 new cases of homozygous β-thalassemic patients was seen at first presentation, before any treatment.[2] From this cohort a good overview of the natural history of the disease can be drawn up. It has also allowed us to investigate some phenotypic data concerning hemoglobin expression.

Clinical Presentation

Most of the cases observed were of the severe form, starting early in life. The first clinical manifestations were observed at the age of 6 months or even before in 25 cases (50%) and between 6 months and one year in an additional 13 cases. However, in a limited proportion of cases—5 in our series of 50—the onset of clinical symptoms was clearly late, between 3 and 5 years of age; and similar additional cases have occasionally been observed. It is interesting to note that in Algeria such late cases are mostly β^0-thalassemias.

The symptoms observed are the classical ones: pallor was always seen, and jaundice and asthenia were present in some but not all of the patients. Splenomegaly was constant after the age of 6 months, as were progressive skeletal changes and the characteristic facial appearance, the degree of which was roughly proportional to the delay between the actual onset and the time of consultation.

Synthesis of Hemoglobin

Hemoglobin production has been studied at different levels. Very broadly dispersed values were observed at first presentation, ranging from 2 to 10 g/dl. It is notable that the higher values were observed both in older children and in babies. β^+ or β^0-thalassemias were distinguished either by isoelectrofocusing before any treatment or by biosynthetic studies: we observed 31 cases of β^+ and 19 cases of β^0-thalassemia.

Special attention has been focused on the expression of fetal hemoglobin, estimated in absolute values g/dl, and not in percentages. In our series we found clearly different values between the two groups: 5.98 ± 1.30 g/dl in β^0-thalassemia and 4.66 ± 1.23 g/dl in β^+-thalassemia ($p < 0.001$) (FIG. 1). Distribution of the experimental values shows few cases with values below 4.5 g/dl in the first group (β^0), and in the second group (β^+) only one case was above 7 g/dl. The difference is still more evident when each group is subdivided between children below 12 months of age and children above this age. In the older children, the values are dispersed in both groups, and the mean value for each group is not very different. In contrast, there is a major difference between the younger children of the two groups. Very low values are observed in the infants presenting with an early onset of β^+-thalassemia; this is not the case in β^0-thalassemia. It is as if in the latter case the delay for the switch from fetal to adult hemoglobin was retarded.

It has been estimated that in the case of severe β-thalassemia a 30-fold expansion of the erythron can account for up to 4–5 g/dl of fetal hemoglobin following the selective survival of the F-cells.[3] Most of the values observed during the first year of life in children with β^+-thalassemia fall within this limit, whereas the values range from 5 to 8 g/dl in cases of β^0-thalassemia. Results are different in children seen after one year of age. In both groups, roughly half of the patients present values below 5 g/dl, and in both groups there are children presenting with apparent overexpression of fetal hemoglobin.

Production of fetal hemoglobin was also explored in transfused children from results obtained immediately before the monthly transfusion. As expected, fetal hemoglobin synthesis was most often depressed, usually below 1 g/dl. In a few cases, however, it was regularly maintained between 2.5 and 3.5 g/dl. Individual cases where a truly high expression of fetal hemoglobin was found were further explored, and a comparison was made with the genotypic data. In these cases two restriction fragment length polymorphism (RFLP) subhaplotypes were found predominantly;

these will be discussed later. However, this association was not an absolute rule, and there are exceptions.

The globin chain composition of fetal hemoglobin was also determined, and here the results fall obviously into two groups according to the 5′ subhaplotypes: the percentage of $^{G}\gamma$ in the first group is between 32 and 55%, and in the second one between 62 and 80%.

A tentative estimation of the synthesis of hemoglobin A_2 was also made, in pg/cell, despite the known cellular heterogeneity. In most of the cases this synthesis appears grossly normal between 0.5 and 1 pg/cell. We observed clearly higher values in four cases only: in all these cases, patients were young babies with severe forms of β^+-thalassemia and low synthesis of Hb F.

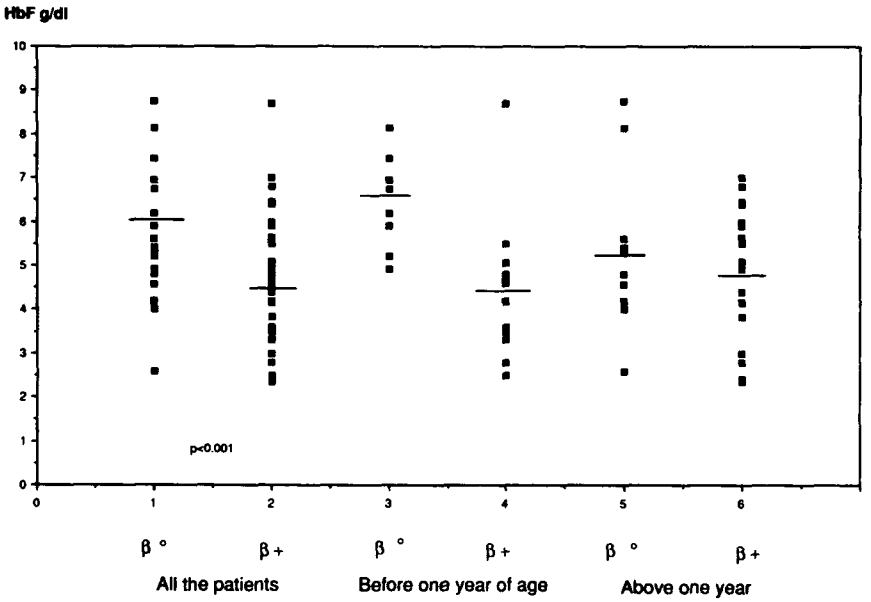


FIGURE 1. Synthesis of fetal hemoglobin (g/dl) by thalassemic patients, as observed at first presentation. **Lines 1 and 2:** all the patients in the two groups, β^0- and β^+-thalassemics. **Lines 3 and 4:** patients seen before one year of age. **Lines 5 and 6:** patients seen after one year of age.

Results observed among siblings in ten families where more than one child presented the homozygous form have allowed us to demonstrate that the expression of hemoglobins F and A_2 is extremely similar within a family, whereas other symptoms clearly depend upon the timing of the evolution of the disease.

GENOTYPIC STUDIES

From the beginning, this study has been intended to determine both the thalassemic mutation and the genetic background surrounding it. Results in these two fields were often obtained simultaneously, following the development of technol-

ogy and the progress of knowledge in the field. We first used restriction mapping for haplotype analysis, detection of some molecular defects, and correlation between genotypic data and phenotypic expression. The availability of oligoprobes, and of the polymerase chain reaction (PCR), has been a major improvement for both series of results. For practical reasons we will present the data on the mutations and their genetic background in two separate sections.

Spectrum of β-Thalassemic Mutations in Algeria

Mutations have been investigated in 88 patients presenting with Cooley's anemia or a thalassemia intermedia syndrome. In the beginning of our investigations, the mutation was identified at the protein level in two cases, one case of hemoglobin Lepore Boston[4] and one of hemoglobin Knossos.[5] In these two cases the other chromosome has not been examined. Investigation of the 86 remaining cases represents 171 chromosomes. The majority of these patients come from a wide area in the vicinity of Algiers or from Kabylia, but minor groups were from the east of the country or were living in France. In families where one or more siblings were followed, only one of them was taken into account.

Results have been obtained by restriction enzyme analysis, cloning and sequencing, and more recently by PCR amplification and slot-blot identification using allele-specific oligonucleotide (ASO) probes. The latter method was the most efficient for a general evaluation. The results can be summarized as follows.

1. There was an exceptionally high proportion of true homozygotes: 47 of our patients—more than half—were found to carry the same identified mutation on both chromosomes.
2. There was no predominant mutation. Four mutations were frequent: G→A at IVS-1 nucleotide (nt) 110;[6] C→T, creating a nonsense codon at codon 39;[7] a frameshift due to a microdeletion of one nucleotide at codon 6;[8,9] and G→A at IVS-1 nt 1.[10] A fifth mutation was clearly less frequent: T→C at IVS-1 nt 6.[11] These results are summarized in TABLE 1. The four most frequent mutations accounted for 83.8% of the cases, and by adding the fifth most frequent one, T→C at IVS-1 nt 6, for 87.3%. These cases are readily accessible to antenatal diagnosis at the DNA level.
3. The molecular heterogeneity of thalassemic mutations is even more evident in the 12% of the cases not belonging to these five major categories. Many of these chromosomes are still under investigation. Some others have been characterized either as previously described mutations—Hb Lepore, Hb Knossos—or as novel ones. Two of these molecular defects have been extensively studied.

In one case, we observed in three siblings of a family a homozygous form of β^+-thalassemia due to a G→A mutation at nt 5 within the IVS-1 donor site consensus sequence.[12] Several mutations have been described at this site, and in all cases, including ours, the production of normal mRNA and β-globin is much lower than it is in cases of mutation of nt 6, confirming the importance of this G residue in mRNA splicing.[13] Nevertheless, the three children we observed presented initially and for several years with a thalassemia intermedia syndrome, this condition tending later on to deteriorate. In the three children a high expression of fetal hemoglobin was found, either at first presentation (6 to 7 g/dl when they were 5–6 years old) or under the

transfusion regimen. In our series of thalassemic patients the 5′ subhaplotype surrounding the γ genes (− − − +) was always associated with this overexpression. This overexpression could be produced by a determinant reacting to erythropoietic stress, or it could be associated with a very long delay in the fetal-to-adult hemoglobin switch.

In another adult patient presenting with thalassemia intermedia and living a subnormal life, a mutation within the IVS-2 acceptor site consensus sequence was found on one chromosome; the other chromosome carried a frameshift mutation and was β^0-thalassemic.[14] This novel mutation, which still allows the production of a sizable amount of β chain, has permitted us to study the functional role of the polypyrimidine tract. The presence of a mutation within this sequence mostly causes a reduction in the quantity of mRNA produced; there are also trace amounts of abnormally spliced RNAs (data, submitted for publication). Observation of this patient emphasized the fact that her relatively good condition was supported by an extremely high peripheral erythroblastosis, raising the possible role of growth factors in the clinical presentation.

TABLE 1. β-Thalassemic Mutations in Algeria

Mutations[a]		No. of Chromosomes	%		
Frequent					
Codon 39 (nons)	C→T	45	26.0	83.8	87.3
IVS-1 nt 110	G→A	43	25.4		
IVS-1 nt 1	G→A	25	14.5		
Codon 6 (−A)	G—G	31	17.9		
IVS-1 nt 6	T→C	6	3.5		
Others					
IVS-1 nt 5	G→A	2		2.9	12.7
IVS-2 nt 843	T→G	1			
Hb Lepore Boston		1			
Codon 27	G→T	1			
Not identified		17	9.8		
Total		172	100		

[a]nons, nonsense codon; nt, nucleotide; Hb, hemoglobin.

Genetic Environment of the Thalassemic Mutations

Even before identifying the thalassemic mutations, we had started to define RFLP haplotypes using the classical seven restriction sites described by Orkin[10] in Mediterranean populations.[15] Initially this study was progressively extended to more patients in parallel with family studies in order to evaluate the degree of feasibility of antenatal diagnosis in this population.[16] The results can be summarized as follows.

1. Previously described haplotypes were found in the Algerian population, but in proportions different from the ones observed formerly. A relatively high frequency of haplotype IX was found and, to a lesser degree, haplotype III. Four novel haplotypes were also demonstrated.
2. In more than 70% of the cases the restriction pattern of the 3′ part of the cluster was identical, and by extrapolation the framework was assumed to be of type I only.

3. In this haplotype analysis, as in the mutation identification, the proportion of individuals carrying the same haplotype on both chromosomes was extremely high, more than one-half. A similar feature was found in a sizable although smaller proportion of the parents, making antenatal diagnosis by this method unfeasible in such cases.

Study of the γψβ Subhaplotype

Our attention has initially been focused on the occurrence of thalassemia intermedia. Since no α-thalassemia was found, and patients were β^0-thalassemic, the mildness of the disease was clearly related to the overproduction of fetal hemoglobin, between 8 and 10 g/dl in the first series. All the patients carried the same haplotype IX on both chromosomes with a C→T mutation at position −158 5′ to the cap site of $^G\gamma$. In these cases too, the proportion of $^G\gamma$ chain was found to be between 70 and 80%, similar to what is seen during the fetal stage of ontogenesis. Of special interest was the fact that a similar amelioration of the clinical condition was observed in homozygous sicklers from Senegal carrying the same haplotype but whose disease obviously resulted from a different pathophysiological process.[17,18] The same observation has now been extended to a larger series and also reported by other authors.[19]

In thalassemic children who are compound heterozygotes for haplotype IX and another haplotype, such a clinical amelioration is inconstant. It is nevertheless frequently observed at first presentation: the clinical onset of the disease often seems to be delayed to later than 3 years of age, and the decay of fetal hemoglobin synthesis has been delayed over several years in most of the cases of our series. In the same children, the production of $^G\gamma$ chain is maintained to a level of over 60% of the total synthesis of fetal hemoglobin, and the $^G\gamma/^A\gamma$ ratio is clearly dissociated. All these results seem to establish a strong relation between a mode of expression of fetal hemoglobin and a defined haplotype.

This relation was assumed to be due to the 5′ haplotype directly surrounding the γ genes. Therefore attention was first given to haplotype III, which apparently shares with haplotype IX the same battery of polymorphisms in the γψβ zones, including the C→T mutation at position −158 5′ to the $^G\gamma$ cap site. This haplotype is a rather common one in Algeria, but in none of the patients presenting a chromosome carrying this haplotype, even in the homozygote condition, was an overexpression of fetal hemoglobin observed. The existence of some other structural difference involving a potentially regulatory sequence was further supported by our simultaneous study of individuals of Italian origin who were homozygous for the same haplotype III but were carriers of a determinant for hereditary persistence of fetal hemoglobin (HPFH). A comparative study was then performed that focused on the γ gene promoters on one hand, but also on the putative enhancer located 3′ to the $^A\gamma$ gene. A series of mutations within this enhancer had recently been described in association with HPFH Seattle, and it had been suggested that they might have a role in this phenotype.[20] These three mutations were demonstrated to exist both in individuals with (Italian) and without (Algerian) HPFH, as well as in other individuals presenting other phenotypes. Their presence in an Algerian thalassemic patient without any overexpression of fetal hemoglobin definitely ruled out their involvement in the high expression of the γ genes.[21,22]

Two other 5′ subhaplotypes should be mentioned with respect to their relation with this phenotype. As mentioned earlier, we have observed an overexpression of fetal hemoglobin, at least for several years in the beginning of life, in patients homozygous for the 5′ subhaplotype − − − − +. The same type of overexpression was

observed with a thalassemic intermedia syndrome in a patient who is a compound heterozygote carrying this subhaplotype on one chromosome. Up to now no precise determinant has been identified, but it should be noted that this subhaplotype is the same as the one observed in the Benin type β^S chromosome.[23] Our results suggest therefore that, when submitted to a strong erythropoietic stress, this subhaplotype could be linked to determinants responsible for a true overexpression of fetal hemoglobin.

In all the patients homozygous for haplotype II, the proportion of $^G\gamma$ chain synthesis ($^G\gamma/^A\gamma$) was tightly clustered between 60 and 65%, but without global overexpression of fetal hemoglobin. In similar cases it has been suggested that a 4-nt deletion 5′ to the $^A\gamma$ gene could be responsible for decreased expression of this gene and consequently for a relative overexpression of the $^G\gamma$ gene.[24] Most of our patients were investigated for this mutation; it was found in some, but not all, of the cases (unpublished observations).

TABLE 2. Linkage Disequilibrium between Mutations and Haplotypes in 121 Chromosomes

	Mutation[a]					
Haplotype	IVS-1 nt 110 (G→A)	Codon 39 (nons) (C→T)	IVS-1 nt 1 (G→A)	Codon 6 (FS) (−A)	IVS-1 nt 6 (T→C)	Rare
+−−−− ++ (I)	32	12	1	1		
−++−+ ++ (II)	2	22				
−+−++ +− (III)			6			
+−−−− +− (V)			14			
−++−− −+ (VI)					4	
−+−++ ++ (IX)		1	2	17		
−−−−− ++ (A)				1		
−++−+ +− (B)			1			
−−−−− +− (C)						2
−−−−− −+ (D)					2	1
Total	34	35	24	19	6	3
Linkage with the major haplotype	94.1%	62.8%	58.3%	89.5%	66.6%	

[a]nt, nucleotide; nons, nonsense mutation; FS, frameshift.

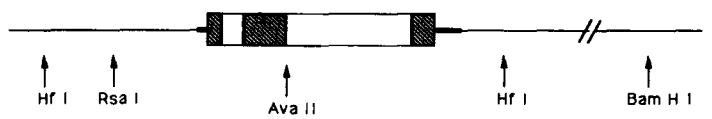
Further Study of the 3′ Subhaplotype

As with the identification of mutations, the study of haplotypes was drastically modified by the use of PCR. The 3′ part of the haplotype is usually determined by only two restriction enzymes, *Ava* II—which potentially cleaves in the β gene IVS-2—and *Bam*H I—which cleaves about 8-kb downstream of the *Ava* II site. The majority of the chromosomes we studied were + for *Ava* II and *Bam*H I, leading us to extrapolate from this data an intragenic structure belonging to framework I, which is common to haplotypes I, II and IX.[10] A comparison between these results and the mutations mentioned above (TABLE 1) shows that the linkage between mutation and haplotype varies greatly from one mutation to another (TABLE 2). It can be seen that the mutation at IVS-1 nt 110 is consistently associated with haplotype I with only two exceptions, which can be explained by recombination events.[25] The second frequent mutation, nonsense codon 39, is found rather frequently with other associations than

the classical one, haplotype II, but here too a recombination event may explain the observed data. The same is true again for the association of haplotype IX with the frameshift at codon 6. The mutation at IVS-1 nt 6 is infrequent, but the same type of explanation remains valid: all of the data are consistent with the presence of a mutation on a single framework. However, for the mutation at IVS-1 nt 1, not only is the classically described[26] linkage with haplotype V not consistently seen (14/24: hardly more than half the cases), but in three cases the haplotypes observed suggest the presence of a different framework.

The same variable association has been described for Sardinians with a nonsense mutation at codon 39.[27] When the different haplotypes observed share a similar 3′ part, the simplest explanation is a recombination event. On the other hand, when differences in this 3′ part are observed, the question of a recurrent mutation can be raised.

In a similar way the pluricentric origin of the β^S mutation was first proposed on the basis of its presence on chromosomes bearing various haplotypes.[23] It was more



Mutation						n
IVS 1 nt 110 G → A	+	−	+	+	+	9
Co 39 C → T	+	−	+	+	+	16
IVS 1 nt 1 G → A	+	−	+	−	−	16
IVS 1 nt 6 T → C	+	−	−	+	+	3
Co 6 (Del A)	+	+	+	+	+	8

FIGURE 2. (Upper panel) The newly defined 3′ subhaplotype, including five restriction polymorphisms. **(Lower panel)** The linkage disequilibrium most commonly observed between the thalassemic mutations frequent in the Algerian population and the various subhaplotypes. Hf I, *Hinf* I site; Co, codon; Del A, deletion of an A.

recently confirmed by a structural study of sequences located 5′ to the β gene itself, which were demonstrated to be polymorphic and specific for each origin of the β^S mutation.[28a]

We therefore decided to apply a similar approach to the study of the β-thalassemic DNAs. Our hypothesis was that, for a more detailed investigation of the genetic background surrounding β-thalassemic mutations, as a first step determination of the sequence could be replaced by assay of selected restriction polymorphisms, provided they were located on both sides of the β gene and close enough to probably preclude a recombination event. Given the size of the fragments obtained, the presence of these polymorphic sites might be ambiguous on genomic DNA, but easy to assess on an amplified segment.

The newly defined haplotype involves the following restriction sites: 5′ to the β gene, the *Hinf* site at −1.1 kb and the *Rsa* I site at position −550;[29] within the gene itself, the *Ava* II site; 3′ to the β gene, the *Hinf* I site close to the poly(A) attachment site,[30] and the *Bam*H I site 8 kb downstream. This haplotype has been explored in

TABLE 3. Atypical Cases Observed

Case	Haplotype	Mutations[a]	Polymorphisms 5′ to the β Gene		Polymorphisms 3′ to the β Gene	
			Hinf I	*Rsa* I	*Hinf* I	*Bam*H I
1	I/I	IVS-1 nt 110/IVS-1 nt 110	+/+	+/−	+/+	+/+
2[b]	II/IX	Nons 39/Nons 39	+/+	−/−	+/+	+/+
3	II/V	Nons 39/IVS-1 nt 1	+/−	+/−	+/−	+/−
4	IX/IX	IVS-1 nt 1/IVS-1 nt 1	+/+	−/−	−/−	+/+
5	I/I	IVS-1 nt 110/IVS-1 nt 1	n.d.[c]	n.d.[c]	+/−	+/+
6	II/B[d]	Nons 39/IVS-1 nt 1	+/+	n.d.[c]	+/+	+/−

[a]Nons 39, nonsense codon 39; nt, nucleotide.
[b]In case 2, all the sites are homozygous, including *Rsa* I, consistent with the presence of the nonsense codon 39 homozygous mutation. Haplotype IX is therefore not the usual one, but probably a recombinant (IX in 5′, II in 3′).
[c]n.d., not determined.
[d]B is the name arbitrarily given to the haplotype −++−+ +−.

most of the chromosomes for which the thalassemic mutation had been identified. It gives consistent results, which are shown in FIGURE 2. It should be noted that the *Rsa* I site seems to be specifically present in association with haplotype IX and the frameshift mutation at codon 6. Since haplotypes I, II, and IX are classically associated with framework I, the existence of a consistent difference in the 3′ subhaplotype requires a further identification of polymorphisms not detectable by restriction enzymes.

Nevertheless, some exceptions are found to the linkage disequilibrium generally observed (TABLE 3). They can be divided into two groups. In cases 1 to 3, a discrepancy was found in the sites located 5′ to the β gene. The *Hinf* I site was previously demonstrated to lie within a region of relative sequence randomization.[31] Our results could suggest that this zone includes the *Rsa* I site and therefore extends extremely close to the β gene. The other three cases (4 to 6) raise a more difficult question. All these cases represent chromosomes which carry a mutation at IVS-1 nt 1 and in which no linkage disequilibrium is observed between the *Hinf* I site 3′ to the β gene and the *Bam*H I site.[10] Since the nature of the framework is usually extrapolated from the presence or absence of the *Bam*H I site, the framework was further identified by sequencing IVS-2 in two ambiguous cases (4 and 6). In both cases, the *Hinf* I site was found to be a better index of the framework than was the *Bam*H I site (TABLE 4).

This raises the question of the reliability of this *Bam*H I site located 8 kb 3′ to the

TABLE 4. Atypical Cases Testing the Validity of the *Bam*H I Site: Determination of the Framework by Sequencing

Case	Haplotypes[a]	Mutations[b]	Restriction Polymorphisms			IVS-2	
			Ava II	*Hinf* I (3′ to β)	*Bam*H I	nt 74	Framework[c]
4	IX/IX	IVS-1 nt 1/IVS-1 nt 1	+/+	−/−	+/+	T/T	II/II
6	II/B	Nons 39/IVS-1 nt 1	+/+	+/+	+/−	G/G	I/I

[a]B is the name arbitrarily given to the haplotype −++−+ +−.
[b]nt, nucleotide; Nons 39, nonsense codon 39.
[c]Framework as determined by sequencing for nt 74 of IVS-2.

β gene. A technical problem seems to be ruled out since in both cases the site was present. Recombination between the β gene and this restriction site can be invoked as an explanation, but it also has to be remembered that the *Bam*H I site lies within an L1 sequence. Such sequences, which resemble pseudogenes, could be the target for genetic events such as gene conversion and may tend to homogenize more than other sequences.[32] The last case (6) is also the only one in our series which raises the issue of having the same mutations (IVS-1 nt 1) on two different frameworks (I and II) in the same population, and therefore of a recurrent mutation or a gene conversion.

From an anthropological point of view, some data can be generalized. Two mutations, namely IVS-1 nt 110 and the frameshift at codon 6, present a very high degree of linkage with all the polymorphisms, which could indicate a relatively recent origin or introduction into the country. The nonsense codon 39, found everywhere in the Western part of the Mediterranean, is found associated with many recombination events. Interestingly, the IVS-1 nt 1 mutation is found to have the weakest linkage to genetic background and is associated with various genetic events. During a first investigation of the available cases, they were all found to originate either from Kabylia or from the south of the country. This could indicate a very ancient and possibly local origin of the IVS-1 nt 1 mutation. From this point of view it is interesting to compare our data with the various results obtained in Mediterranean populations[33] and especially in that of neighboring Tunisia.[34] One finds in Algeria roughly similar quantities of chromosomes carrying the nonsense codon 39 mutation which is common to the Latin world and the IVS-1 nt 110 G→A mutation from the Eastern part of the Meditteranean. The frameshift mutant (−1 at codon 6) seems to be generally distributed in North Africa, and it is amazing to observe that, outside of Algeria, the IVS-1 nt 1 G→A mutation is frequent mainly in Portugal.[35]

CONCLUSION

In conclusion, a systematic investigation of β-thalassemia in Algeria has given valuable information concerning the spectrum of the thalassemic genes in the country and has demonstrated the existence of novel mutations. Because there was access to patients not yet treated, this investigation gives information concerning the expression of fetal hemoglobin. The very heterogenous distribution has also allowed the study of the linkage between mutations and the genetic background in which they arose.

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Metabolism of Non-Translatable Globin mRNAs Arising from Premature Translation Termination Codons

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INTRODUCTION

β-Thalassemias characterized by a complete absence of β-globin synthesis are called β^0-thalassemias; as a group they constitute approximately one-third of all cases of β-thalassemia.[1-3] The most common mutation giving rise to defective translation is a base substitution that alters a codon that specifies incorporation of an amino acid into one which is a translation stop-codon (translation termination codon, "nonsense codon"). As a result, ribosomes translating the mutant mRNA species encounter a translation stop signal prematurely and dissociate from the message. These mRNAs can encode only prematurely terminated peptide fragments that cannot function as β-globin molecules. Therefore, no β-globin is produced from the activity of the affected allele.

We[4] and others[5,6] observed that patients with β^0-thalassemia due to premature translation termination of their β-globin mRNA exhibit reduced amounts of β-globin mRNA in their erythroid cells, even though one might expect this mRNA to be afflicted only with deranged function, rather than defective accumulation. Several hypotheses have been advanced to explain this phenomenon. Central to almost all of these proposals has been the assumption that non-translated mRNAs are unstable in the cytoplasm, presumably because they are incapable of receiving full protection by polyribosomes against nuclease degradation. However, we[7] and others[8] demonstrated that no detectable abnormalities of β-globin mRNA stability existed in the cytoplasm of erythroid cells from these patients or in non-erythroid cells transfected with the β^0-thalassemia genes. More recently, we have also shown[9] that synthetic transcripts generated *in vitro* by coupled transcription-translation are as stable as normal β-globin mRNA, even when incubated with a variety of cell extracts under conditions that cause 50-fold variations in absolute mRNA stability. Yet, the lesion in mRNA accumulation must arise from the process of premature translation termination, because mutations introduced at a particular position of the β-globin mRNA cause reduced accumulation only when they create translation stop-codons.[10] Taken together, these observations suggest that translation termination causes reduced mRNA accumulation by means other than cytoplasmic instability of the

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mRNA. Indeed, the weight of current evidence favors a lesion in an earlier, intranuclear step of RNA metabolism.

During the past three years, our observations regarding β^0-thalassemia have been extended by others, who have shown that the phenomenon of reduced mRNA accumulation due to premature translation termination appears to be a general phenomenon.[11-13] Indeed, Urlaub and co-workers[13] have observed that dihydrofolate reductase mutants carrying translation termination codons in the last exon exhibit normal mRNA accumulation, whereas mutations in any of the antecedent exons result in defective accumulation. This provides indirect but persuasive evidence that

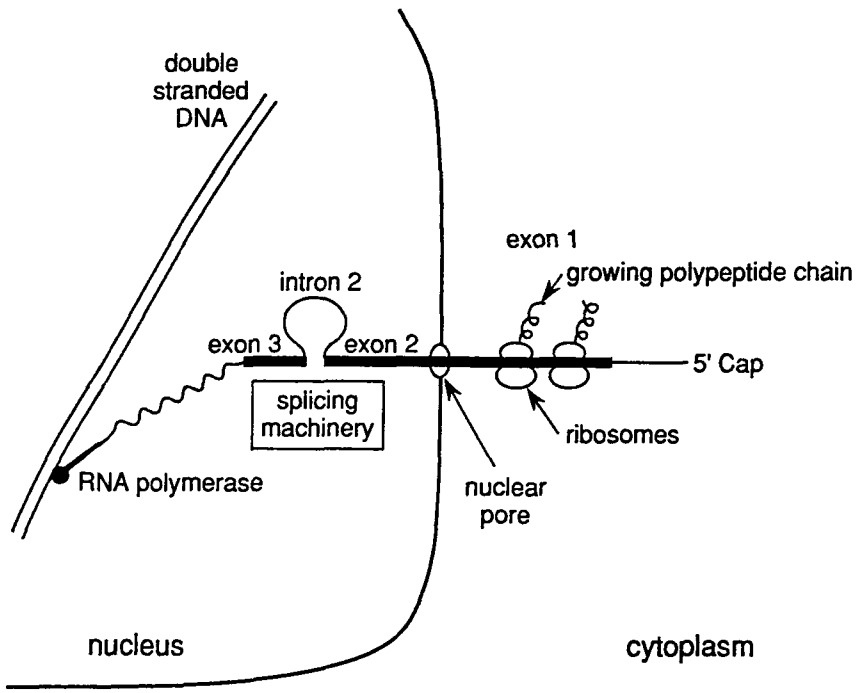

FIGURE 1. Proposed model for mRNA transport out of the nucleus by a mechanism which is coupled with the splicing and translation processes. During translation of mRNA, the ribosomes pull the mRNA through the nuclear pore as it emerges from the nucleus, pulling it through the splicing mechanism at the same time.

mRNA splicing and mRNA translation might be coupled, perhaps by the mechanism diagrammed in FIGURE 1. According to this model, mRNA precursors are spliced upon topologically constrained structures within the nucleus; the newly processed exons are "spooled" through the nuclear pore by a process that involves both binding to ribosomes on the cytoplasmic face of the pore *and* a round of mRNA translation.

Premature translation termination would "stall" the spooling process, resulting in arrested splicing and, possibly, degradation of the mRNA precursor within the nucleus. Premature translation termination in the last exon would have no impact, because the splicing reaction would presumably be complete before the cytoplasmic

ribosome encountered the premature stop signal. As part of our effort to test this model in the case of β-thalassemia syndromes, we have asked whether non-translated β-globin mRNAs can associate with polyribosomes. Our results are summarized in this report.

MATERIALS AND METHODS

For these experiments, cloned β-globin genes were introduced into pGEM plasmid vectors as described. The pGEM vectors carry the promoter and transcription start sites for microbial RNA polymerases, thus permitting cell-free transcription of the inserted recombinant DNA sequence into full-length mRNA. The transcription reactions were performed in the presence of high-specific-activity [^{32}P]UTP, in order to generate highly radioactive globin mRNA.[9] The mRNAs were then incubated with "CAPping" enzyme in order to add to the transcripts a 5′ CAP, necessary for translational efficiency.[9] After purification by phenol/chloroform/isoamyl alcohol extraction and ethanol precipitation, the CAPped mRNAs were incubated in rabbit reticulocyte cell-free translation extracts (Promega Biotec).[9] At the end of the incubation, the extracts were applied to 15–30% linear sucrose gradients, centrifuged at 36,000 rpm for 4 h, and analyzed by elution through a fraction collector.[14] Individual fractions were mixed with scintillation fluid and counted. The pattern of the counts obtained demonstrated the presence or absence of ribosomes and the predominance of particular polyribosomal components. Unbound RNA and RNA bound to 40S subunits remained near the top of the gradient, whereas RNA associated with 6–7 ribosomes migrated very close to the bottom.

A number of the sucrose gradient profiles were further examined for the presence of structurally intact radioactive globin mRNA. After recovery of the RNA from aliquots of each fraction by ethanol precipitation, this RNA was analyzed by electrophoresis on 1.2% agarose–formaldehyde denaturing gels.[9] In all experiments discussed in this report, structural integrity of the globin mRNA was confirmed by the presence of only the full-length 650-base band characteristic of intact globin mRNA.

Two globin mRNAs were compared in the experiments described in RESULTS: normal β-globin mRNA, originally cloned from the reticulocyte mRNA of a patient with hereditary spherocytosis,[9] and a non-translatable β^0-thalassemia mRNA carrying an amber translation termination codon (CAG→UAG) at position 39. The cloning and characterization of these globin genes have previously been described.[7] We have previously demonstrated that erythroid cells from the patient with the amber mutation accumulate only 5% of the normal levels of β-globin mRNA; moreover, this mRNA is stable in the cytoplasm and present at very low levels within the nucleus.[7,10]

RESULTS

FIGURE 2 shows the plasmid vector/insert constructs that were prepared for these studies. FIGURE 3 demonstrates that mRNA generated from these templates was functional if the original template was derived from normal β-globin mRNA but incapable of translation if the template was derived from β^0-39 globin mRNA. These results establish the functional integrity of the transcripts generated by *in vitro* coupled transcription-translation.

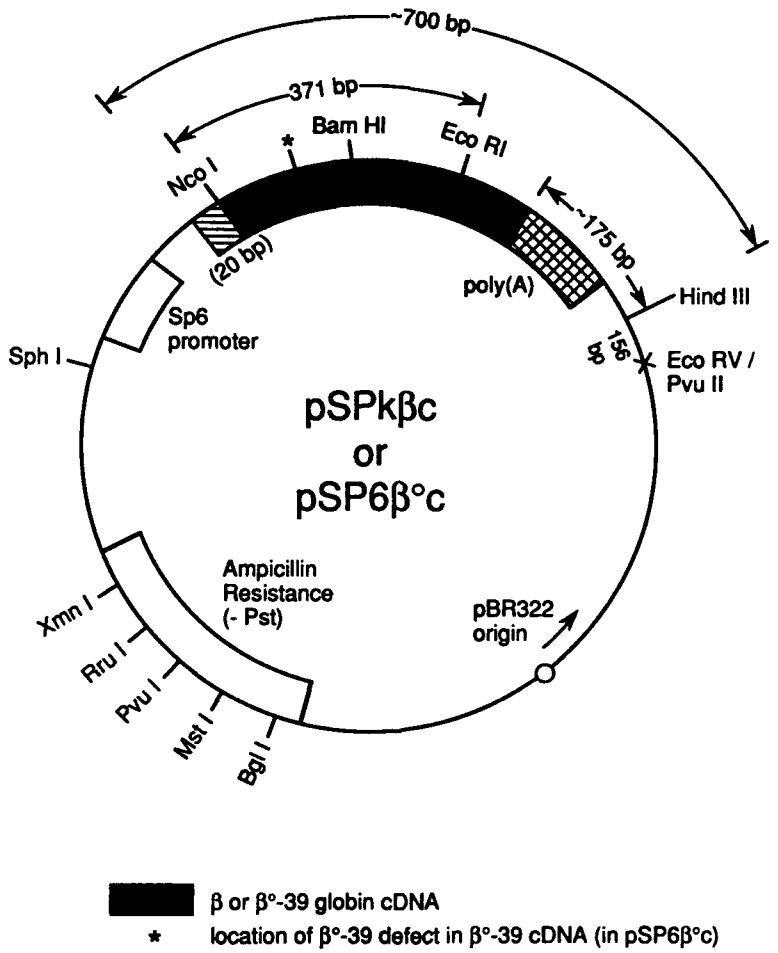

FIGURE 2. The construction of the plasmids pSPkβc and pSP6β^{0}c. These plasmids contain (*shaded region*) the full-length normal β-globin cDNA (pSPkβc) or the β^0-39 globin cDNA (pSP6β^{0}c) and an upstream SP6 promoter sequence which allows the cDNA to be transcribed *in vitro.* In addition, pSPkβc contains a 20-bp 5′ flanking region (*striped box*) and a simian virus 40 (SV40) sequence which codes for a poly(A) tail (*cross-hatched box*).

As shown by the theoretical diagram in FIGURE 4, one would expect normally translated β-globin mRNA to associate with ribosomes in the cell-free translation extract; after a brief (20-min) period of incubation, most of the mRNA should be carried on polyribosomes. The mRNA concentration used in each of our experiments was chosen, on the basis of preliminary experiments, to be rate-limiting. In other words, the amount of translation was directly dependent upon the amount of mRNA added. Under these conditions, one might expect the vast majority of the mRNA molecules to be bound to ribosomes, since other components in the translation mix are *not* limiting.

The size (number of ribosomes/mRNA molecule) of polyribosomes will depend upon the relative rates of initiation, elongation, termination, and dissociation of the ribosomes on the mRNA transcript. Previous work[2] has established that the median size for β-globin mRNA under optimal translation conditions is 3–4 ribosomes/mRNA molecule. In contrast, mRNA blocked by a premature terminator codon occurring at amino acid 39 should "load" only 1 or 2 ribosomes per mRNA molecule, since the ribosomes cannot proceed further downstream than that point. The amount of mRNA sequence to the 5′ side of the blocked termination codon is sufficient to accommodate only 1–2 ribosomes. Therefore, as shown by the diagram (FIG. 4), a smaller modal distribution of ribosome sizes should be encountered, with essentially no polyribosomes accumulating to sizes larger than dimers ("disomes").

As shown in FIGURE 5, the observed results were surprising in view of the aforementioned predictions. Normally translated β-globin mRNA gave the expected result, but β^0-39 globin mRNA also bound efficiently to ribosomes, with a modal distribution quite similar to that of the normal mRNA. Moreover, as shown in FIGURES 6–8, inhibitors of initiation (aurintricarboxylic acid) or elongation (anisomycin or amino acid starvation) of translation yielded the expected alterations in polyribosome profiles. In other words, initiation inhibitors prevented formation of polyribosomes larger than monosomes, whereas elongation inhibitors reduced the average size of the polyribosomes. These results suggest that our system is sufficiently sensitive to detect altered polyribosome profiles due to impaired mRNA translation. Note that FIGURE 8 shows that the β^0-39 globin mRNA responded to inhibitors in a manner essentially identical to that of normal β-globin mRNA. This supports the

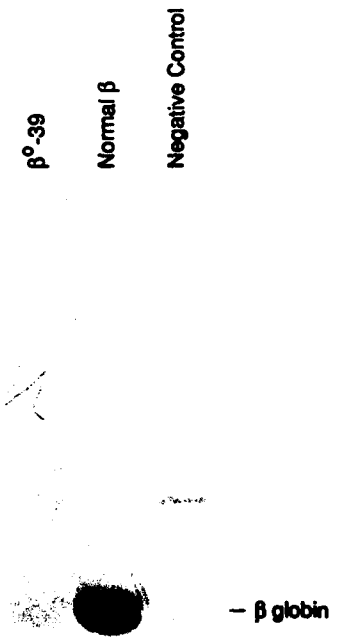

FIGURE 3. Translation of normal β and β^0-39 thalassemia globin mRNAs. To demonstrate the translatability of the synthetic transcripts and the activity of the rabbit reticulocyte lysate, translation products labeled only with [^{35}S]methionine were electrophoresed on a polyacrylamide gel along with a negative control translation assay conducted without any mRNA.

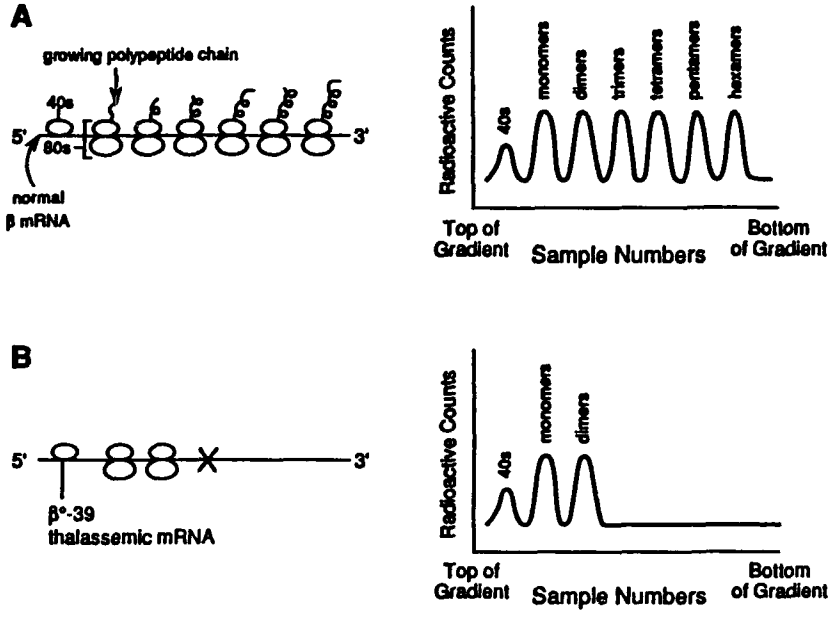

FIGURE 4. The predicted polyribosome profiles of normal β and β^0-39 thalassemia globin mRNAs. Shown are the schematic diagrams of polyribosomes on normal β and on β^0-39 thalassemia globin mRNAs and their respective polyribosome profiles. (**A**) During the translation of normal β-globin mRNA, association of up to 6 ribosomes per mRNA chain is expected. (**B**) In contrast, a maximum of only 2 ribosomes per mRNA chain is expected during the translation of β^0-39 thalassemia globin mRNA because of the nonsense mutation at codon 39.

notion that β^0-39 globin mRNA is associating with polyribosomes in a translation-related manner, rather that by some non-specific mechanism.

DISCUSSION

Our recent studies suggest that non-translatable β-globin mRNAs can associate with polyribosomes, even though normal translation is blocked. We do not yet have information that provides a possible mechanism for this association with ribosomes. However, as shown in TABLE 1, a review of the human β-globin mRNA sequence suggests numerous potential opportunities for re-initiation at in-frame or out-of-frame methionine codons downstream. The maximum size of the polyribosomes that could be formed for each of those initiation sites is indicated in TABLE 1. Of particular note is the potential to utilize the normally occurring internal methionine as an alternate translation re-initiation site. Note that the sequence of nucleotides surrounding this methionine forms a favorable translation-initiation consensus start-site.

Our previous results, coupled with the results reviewed in this communication, suggest strongly that non-translatable β-globin mRNAs can survive normally in the cytoplasm at least in part because of the fact that they have the potential to enter into polyribosomes, even if they cannot be normally translated. The low levels of

accumulation of non-translated globin and non-globin mRNAs must then arise from mechanisms other than cytoplasmic instability. Our current work, and that of several other laboratories, now focuses on intranuclear steps of globin mRNA metabolism. Especially exciting is the possibility that intranuclear mRNA splicing and cytoplasmic mRNA translation may be coupled, or at least cross-regulated, by transnuclear membrane signaling.

SUMMARY

A common cause of β-thalassemia is defective translation of β-globin mRNA. Base substitutions which convert codons that specify incorporation of amino acids

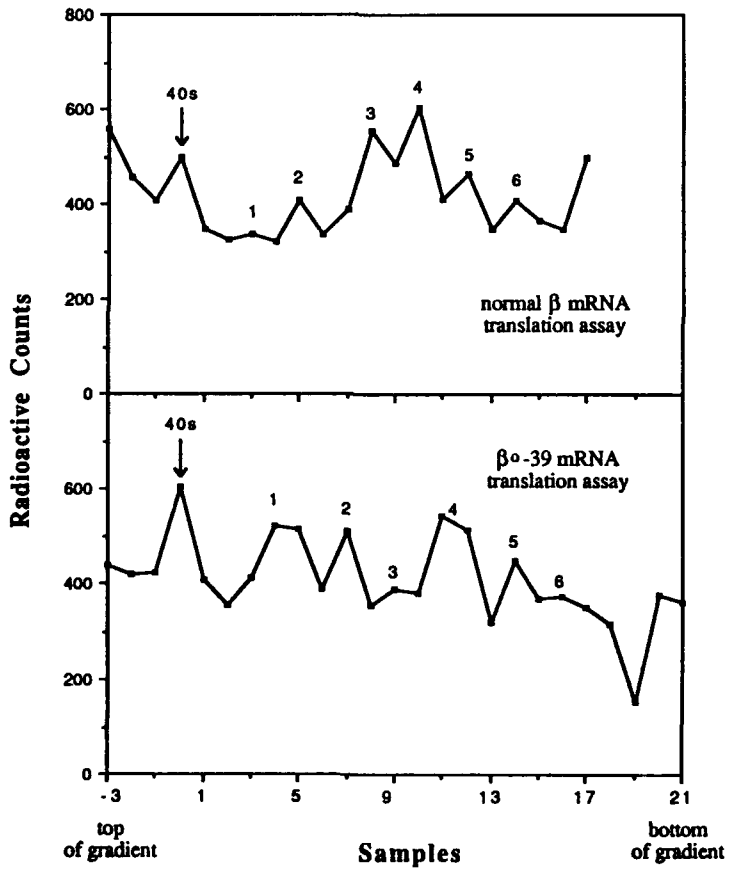

FIGURE 5. Normal β and β^0-39 thalassemia globin polyribosome profiles. The peaks surmised to be due to the 40S-mRNA complexes (*arrows*) have been put in alignment with each other for comparison. The *numbers by the peaks* indicate the ribosome content (1, monomers; 2, dimers; 3, trimers, etc.). Samples which came from above sample 0 in the sucrose gradient are designated by *negative numbers*, while those to the bottom of the gradient are designated by *positive numbers*.

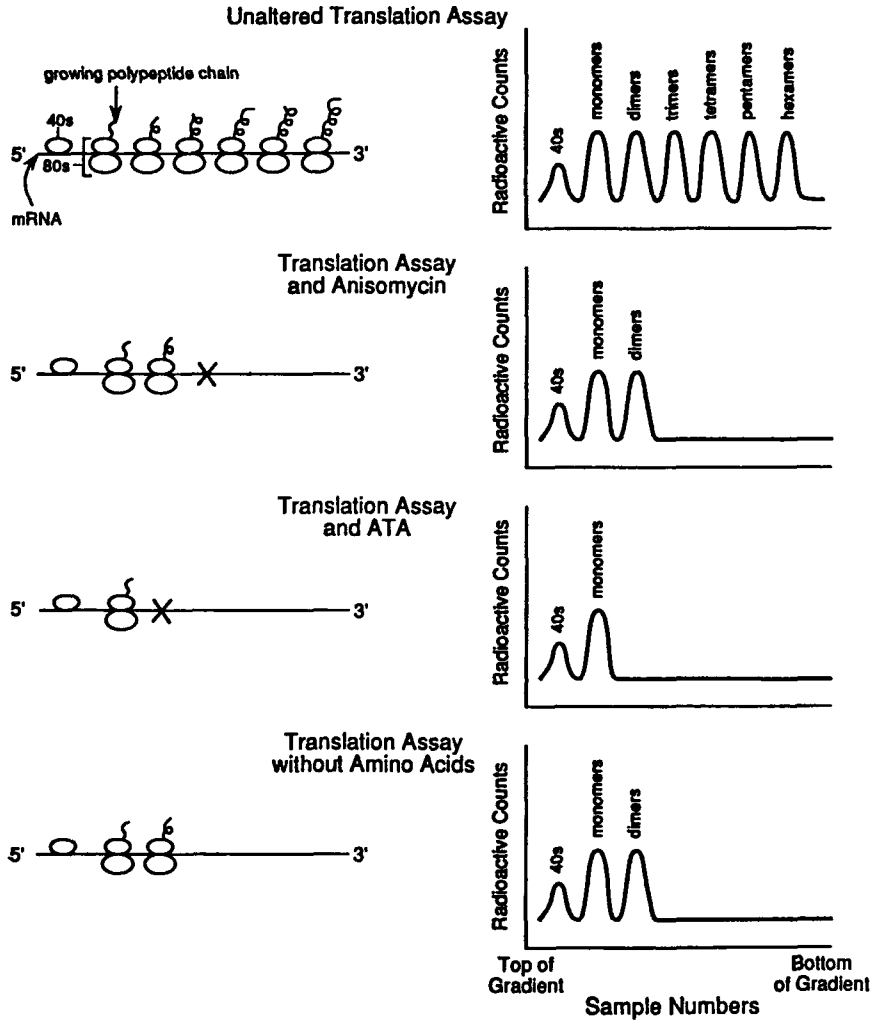

FIGURE 6. Predicted profiles of functional, authentic β-globin polyribosomes with the addition of translation inhibitors or the omission of amino acids. The inhibitors are aurintricarboxylic acid (ATA), which inhibits initiation, and anisomycin, which inhibits elongation. The schematic diagrams of polyribosomes and their respective predicted polyribosome profiles are shown.

into ones that are translation termination codons (nonsense codons) constitute as a group very common causes of β^0-thalassemia. These premature termination codon mutations should cause defective function of β-globin mRNA, rather than inadequate accumulation. Yet, β-globin mRNA in these patients is normally reduced to 0–20% of normal levels. Reduced accumulation has been attributed to instability of the mRNA; by virtue of its presumed inability to be translated on polyribosomes, the

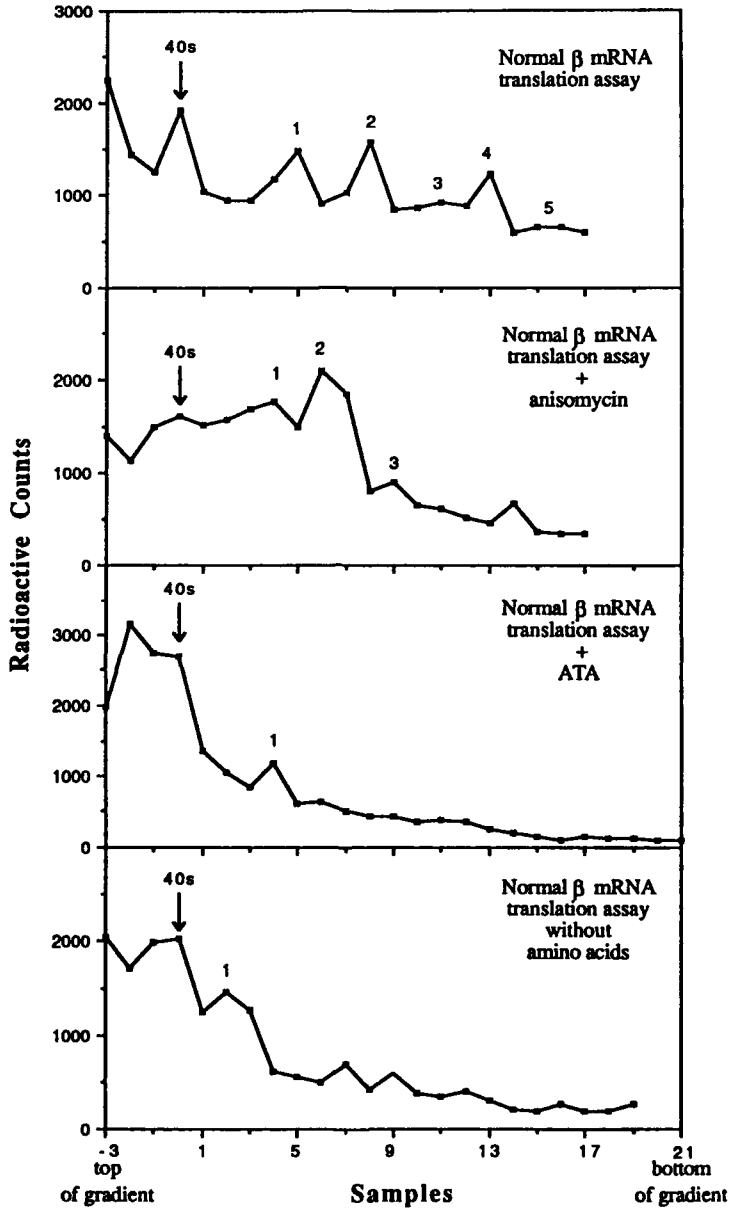

FIGURE 7. The effect of the addition of translation inhibitors or the omission of amino acids on normal β-globin polyribosome profiles. Peaks surmised to be due to 40S-mRNA complexes (*arrows*) have been put in alignment with each other for comparison. The *numbers by the peaks* indicate the ribosome content (1, monomers; 2, dimers; 3, trimers, etc.). Samples which came from above sample 0 in the sucrose gradient are designated by *negative numbers*, while those to the bottom of the gradient are designated by *positive numbers*.

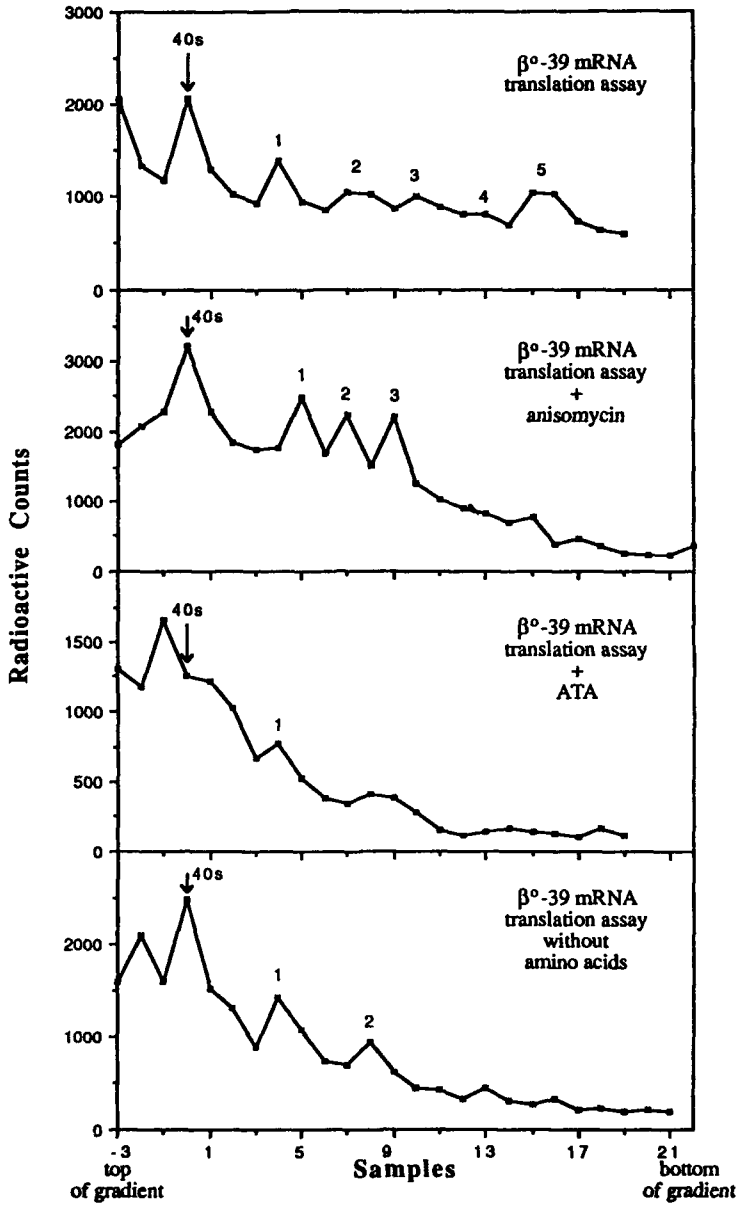

FIGURE 8. The effect of the addition of translation inhibitors or the omission of amino acids on β°-39 globin polyribosome profiles. Peaks surmised to be due to 40S-mRNA complexes (*arrows*) have been put in alignment with each other for comparison. The *numbers by the peaks* indicate the ribosome content (1, monomers; 2, dimers; 3, trimers, etc.). Samples which came from above sample 0 in the sucrose gradient are designated by *negative numbers,* while those to the bottom of the gradient are designated by *positive numbers.*

TABLE 1. Potential Downstream Sites for Re-initiation of Translation of Human β-Globin mRNA and Predicted Maximum Size of Polyribosomes Generated

Position of AUG Site[a]	Flanking Sequence	Position of Termination	Maximum No. of Ribosomes
Codon 55	GUUAUGG	Normal (i.e., 92 codons downstream)	4
Codons 73/74	GUGAUGG	15 codons downstream	1
Codons 63/64	CUCAUGG	25 codons downstream	1
Codons 21/22	UGGAUGA	39 codons downstream	2
Codons 52/53	CUGAUGC	8 codons downstream	1
Codons 139/140	CUAAUGC	18 codons downstream	1

[a]The consensus sequence for initiation is $\overset{-3}{P}NN\underline{AUG}\overset{+4}{G}$. P, adenine or guanine; N, any nucleotide.

mRNA would exist free in the cytoplasm, where it is exposed to nuclease. We have conducted a series of studies on the metabolism of these non-translatable RNAs. We have found that non-translatable β-globin mRNAs are usually stable, and that defective accumulation is due to aberrations in an early intranuclear step of metabolism. We have now shown that these non-translatable mRNAs appear to be capable of associating with polyribosomes despite their inability to undergo complete rounds of translation. These findings argue strongly against hypotheses for reduced accumulation based upon cytoplasmic instability.

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β-Thalassemia Mutations in Sicily[a]

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The thalassemia syndromes are the most common forms of genetic disease in Sicily.[1] An epidemiological survey by the Regional Health Department in 1988 showed 1042 cases of thalassemia major, 336 of thalassemia intermedia, and 194 with β-thalassemia/sickle cell disease.[1,2] The average prevalence of β-thalassemia trait is about 6%, with a heterogeneous distribution ranging from 10% in southeastern Sicily to 3–4% in the north or in the west of the island. From these data we may consider a frequency of 1/270 couples at risk for β-thalassemia and, with 66,000 newborns per year, an annual Cooley's anemia birthrate of 45 new patients.[3] The hematological and genetic heterogeneity of β-thalassemia in Sicily has been widely described.[4–6] In fact, both β^+/β^+ and β^0/β^+ genotypes in Cooley's anemia and in thalassemia intermedia have been found. Moreover, homozygotes for $\delta\beta^0$- or β^0-thalassemia and double heterozygotes, i.e., $\delta\beta^+/\beta^0$ and $\delta\beta^0/\beta^+$ have been described. Furthermore, studies by restriction fragment length polymorphisms (RFLPs) of the β-globin gene cluster showed seven different cleavage patterns; and many of these patients (68.3%) were genetic compounds for different haplotypes, while only 31.7% were haplotype homozygotes.

The enormous progress in the techniques for identification of β-thalassemia mutations has made it possible to characterize a lot of these mutations and to study the types and mechanisms of the molecular defects.[8–10] We have applied these procedures to the study of 300 β-thalassemia chromosomes in our region with the aim of providing the following information: (*a*) characterization of β-thalassemia genes, which is useful for first-trimester prenatal diagnosis by chorionic villi sampling because it makes it possible to perform this procedure in the majority of couples at risk if the number and type of these β-thalassemia molecular defects are known; (*b*) identification of new β-thalassemia genes, which may be used to better understand β-globin gene regulation and, for this reason, to increase our knowledge for gene therapy; (*c*) correlation, if any, between phenotype and genotype, which may improve our management of therapy, especially for thalassemia intermedia patients who, in a lot of cases, show a considerable genetic heterogeneity. Additional goals of our study were to characterize β-thalassemia genes in a single cell, i.e., an oocyte, and in the DNA of trophoblast cells present in the blood of the mother. In the former

[a]This work was supported by the Sicilian Thalassemic Association and CNR Project No. 89.00308.75.

TABLE 1. Frequency of β-Thalassemia Mutations in 300 Sicilian Chromosomes

Mutation	Frequency
B^0 nonsense codon 39	40.1%
IVS-1 nt 110	22.2%
IVS-1 nt 6	18.8%
IVS-1 nt 1	5.5%
IVS-2 nt 745	4.0%
Frameshift at codon 6	2.2%
IVS-1 nt 116	1.1%
Others	6.2%

case the procedure would make it possible to perform preimplantation β-thalassemia prenatal diagnosis. In the latter case the technique could make it possible to avoid the risks involved in fetal tissue sampling.

MATERIALS AND METHODS

Hematological data were obtained using standard procedures.[11] We studied 150 β-thalassemia patients: 86 with Cooley's anemia and 64 with thalassemia intermedia. Moreover, to study the feasibility of prenatal diagnosis we performed DNA analysis in 74 couples at risk. The 300 β-thalassemia chromosomes were studied using oligonucleotides, the polymerase chain reaction (PCR), and direct genomic sequencing. DNA was prepared as described elsewhere. Specific oligonucleotides (19-mers) were chemically synthesized for each of the following mutations: nonsense codon 39 (C → T), IVS-1 nt 110 (G → A), IVS-1 nt 1 (G → A), IVS-1 nt 6 (T → C), IVS-1 nt 116 (T → G), nt −87, frameshift at codon 76. PCR was performed according to Saiki *et al.*[12] β-Thalassemia mutations IVS-2 nt 1, IVS-2 nt 745, and the frameshift at codon 6 were detected by direct restriction endonuclease analysis with *Hph* I, *Rsa* I, and *Cvn* I, respectively, after PCR. Direct sequencing of the amplified product was carried out using third primers end-labeled using [γ-^{32}P]ATP, sequenase, and dideoxynucleoside triphosphates as previously described.[13] The DNA of maternal blood was extracted by standard techniques.[7] After 40 cycles of amplification, using

TABLE 2. Association of β-Thalassemia Mutations and Haplotypes in Sicily

		Frequency					
β-gene Framework	Haplotype	IVS-1 nt 110	β^0 Codon 39	IVS-1 nt 6	IVS-2 nt 745	IVS-1 nt 1	nt −87
1	I	22	15				
	II	1	16				
	IX	3	3				
2	V					3	1
	VI			17			
	VII		1	10	3		
	X			1			

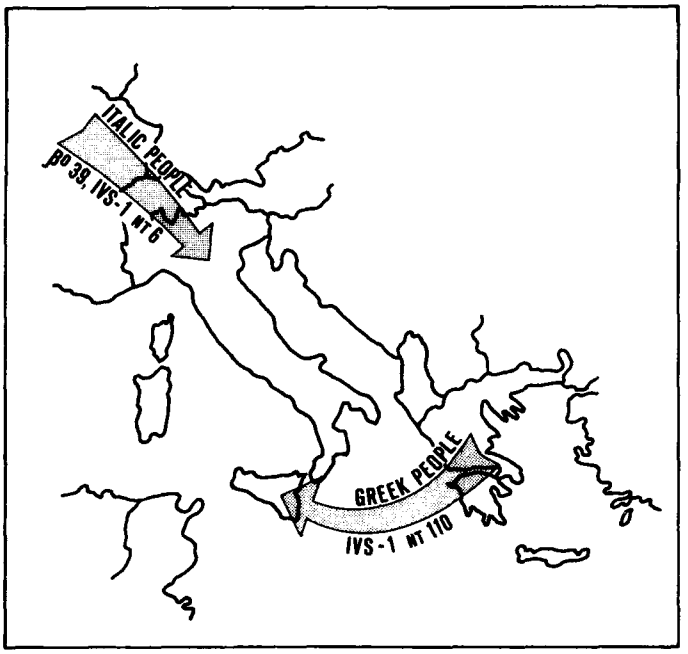

FIGURE 1. Probable origin of different β-thalassemia mutations in Sicily.

the *Taq* polymerase reaction, the samples were reamplified (15–20 cycles) with fresh reagents using nested primers.[14]

RESULTS AND DISCUSSION

The frequency of β-thalassemia mutations is reported in TABLE 1. Our results show that β^0 nonsense codon 39, IVS-1 nt 110, and IVS-1 nt 6 account for 81% of β-thalassemia genes. Other mutations present are IVS-1 nt 1, nt −87, frameshift at codon 6, IVS-1 nt 116, IVS-2 nt 745, IVS-1 nt −1 (codon 30), and IVS-2 nt 1; but the frequency of these was very low, i.e., 0.1% to 5.5%. Two new gene defects, a frameshift at codon 76 and a mutation at IVS-1 nt 130, were found. These results mean that (*a*) in Sicily β^0 nonsense codon 39 is more frequent (40%) than are other mutations and, considering its frequency in Sardinia (91%),[15] the Ferrara area (51.4%)[16] and northern Italy (66%),[17] it is probably the most frequent mutation in

TABLE 3. Feasibility of Prenatal Diagnosis of β-Thalassemia in 74 Sicilian Couples at Risk

Diagnosis Possible	50% Probability of Diagnosis	Diagnosis Not Possible
67 (90.5%)	4 (5.4%)	3 (4.0%)

TABLE 4. Correlation between Genotype and Phenotype in Sicilian Thalassemia Patients

Genotype[a]	Phenotype (%)	
	Cooley's	Intermedia
$\beta^0$39/$\beta^0$39	17	—
IVS-1 nt 110	10	—
$\beta^0$39/IVS-1 nt 110	19	3
$\beta^0$39/IVS-1 nt 6	10	25
IVS-1 nt 110/IVS-1 nt 6	19	22
IVS-1 nt 6/IVS-1 nt 6	—	25
IVS-1 nt 6/IVS-2 nt 745	—	6

$\beta^0$39, β^0 nonsense codon 39.

Italy; (*b*) there is a high frequency of the IVS-1 nt 6 mutation, quite similar to that of IVS-1 nt 110; (*c*) the other mutations are rare in Sicily.

Previous studies showed a high frequency of spreading of the β^0 nonsense codon 39 and IVS-1 nt 6 mutations into two prevalent haplotypes (I and II), in contrast with a low frequency of spreading of the IVS-1 nt 110 mutation (TABLE 2).[18] This could be due to the fact that the two former mutations are older than the latter. We believe that the β^0 nonsense codon 39 and probably the IVS-1 nt 6 mutation may have originated from the Italic populations that came to Italy 3000 years ago,[19] as is suggested by the high frequency of β^0 nonsense codon 39 in different Italian regions and by the presence of IVS-1 nt 6 in other European countries like Portugal,[20] while the IVS-1 nt 110 mutation, present *prevalently in Sicily* and in southern Italy (42%),[16] may have come later from the Greek populations, in which it is very common, especially in Cypriots (FIG. 1).[17]

The presence of three mutations at high frequency explains why 68.3% of the patients that we tested were genetic compounds for different haplotypes. These data make first-trimester prenatal diagnosis possible in more than 80% of Sicilian families (TABLE 3). This value is similar to that found in Sardinia families (91%),[15] using two sets of oligoprobes.

The two new gene defects mentioned above have been characterized after PCR by direct genomic sequencing. The first was a frameshift at codon 76 found in two chromosomes due to the loss of a C residue from codon 76 (nonsense mutation). The second was IVS-1 nt 130, which probably causes a splicing defect.

To show a correlation between genotype and phenotype, the patients were subdivided according to whether they had Cooley's disease or thalassemia intermedia. TABLE 4 shows that patients with β^0 nonsense codon 39 and IVS-1 nt 110 are

TABLE 5. Hematological Data for Various β-Thalassemia Heterozygote Mutations

Mutation[a]	Hb (g/dl)	MCV (fl)	MCH (pg)	A_2 (%)
$\beta^0$39	11.8 ± 1.4	63.5 ± 3.5	20.1 ± 1.2	5.2 ± 0.3
IVS-1 nt 1	10.6 ± 1.2	63.8 ± 4.4	20.4 ± 1.4	4.8 ± 0.3
IVS-2 nt 745	11.8 ± 1.0	65.5 ± 3.3	20.9 ± 1.2	5.3 ± 0.4
IVS-1 nt 110	11.9 ± 1.4	66.6 ± 2.4	21.1 ± 1.0	4.9 ± 0.4
IVS-1 nt 6	13.2 ± 1.5	70.8 ± 2.8	22.5 ± 1.0	3.9 ± 0.3
nt −87	11.5 ± 1.6	70.4 ± 0.9	23.2 ± 0.1	5.5 ± 0.4

[a]$\beta^0$39, β^0 nonsense codon 39.

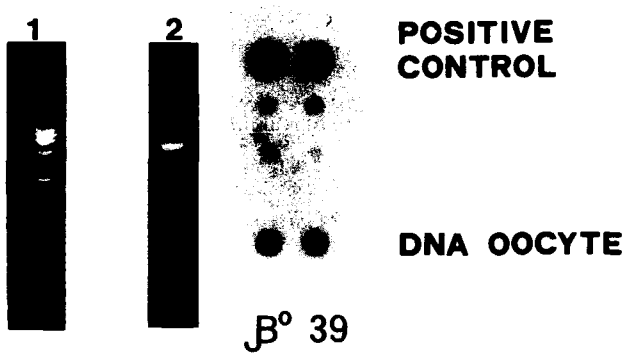

FIGURE 2. Identification of a β^0 nonsense codon 39 mutation from a single oocyte. (**Left panel**) Gel pattern of reamplified oocyte fragment (**lane 2**) and *Hae* III–digested θX174 marker fragments (**lane 1**). (**Right panel**) Dot blot showing hybridization of oocyte DNA with probe for β^0 nonsense codon 39.

more likely to have the Cooley's phenotype, while those with IVS-1 nt 6 usually have thalassemia intermedia. Double heterozygotes for β^0 nonsense codon 39/IVS-1 nt 6 or IVS-1 nt 110/IVS-1 nt 6 can have intermedia or Cooley's disease (TABLE 4). Probably this overlap means that, with today's clinical diagnostic criteria,[21] it is not easy to differentiate Cooley's disease from thalassemia intermedia. According these data, it would be useful for thalassemia intermedia diagnosis to combine the genetic pattern with hematological data, i.e., IVS-1 nt 6 homozygosis and hemoglobin (Hb) > 7 g/dl. Furthermore, the correlation in β-thalassemia heterozygotes between the hematological data and the type of β-thalassemia mutations emphasizes the presence in Sicily of milder β-thalassemia gene defects that may account for thalassemia intermedia (TABLE 5).

In our work towards the other two goals of this study, we were able to identify a β-thalassemia mutation from a single oocyte (FIG. 2); this will make it possible to perform preimplantation prenatal diagnosis. Moreover, it was possible after reampli-

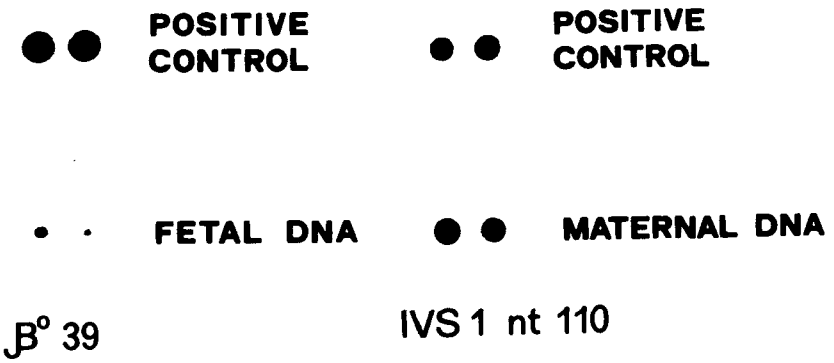

FIGURE 3. Fetal β^0 nonsense codon 39 mutation detected in peripheral maternal blood (**left panel**) and IVS-1 nt 110 mutation detected in maternal DNA (**right panel**).

fication to detect a β-thalassemia mutation of the fetus in peripheral maternal blood containing trophoblast cells (FIG. 3).

The data we have reported in this paper show a molecular basis for genetic heterogeneity of β-thalassemia in Sicily and also will make it possible to perform earlier and safer prenatal diagnosis of hemoglobinopathies.

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A New Strategy for Direct Detection of β-Thalassemia Mutations

Experience of the Créteil Center[a]

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INTRODUCTION

The characterization of β-thalassemia defects in world populations over the past few years has revealed the large molecular heterogeneity of this disease.[1] The development of laboratory techniques based on DNA amplification by the polymerase chain reaction (PCR) has provided a new tool for the rapid detection and characterization of the mutant alleles[2,3] and has enabled their distribution to be studied in relevant populations or in areas where the disease is prevalent.[4-7] We now know that these mutations are population specific, each group carrying a limited number of frequent defects along with other, rare β-thalassemia alleles. Prior knowledge of the spectrum of mutations affecting a given ethnic population greatly facilitates their detection in at-risk individuals or during pregnancy by direct DNA analysis. One of the best techniques is based on the use of allele-specific oligonucleotide (ASO) hybridization to PCR-amplified β-globin DNA sequences. Using this technique, amplified parental DNAs can be screened for the β-thalassemia alleles that account for most of the defects within the population under study. Any mutations undetected in this procedure can be characterized by direct sequencing of the amplified product, a more difficult technique. ASO hybridization is useful in regions of the world where β-thalassemia is caused by a small number of defects or in a population which is homogeneous. In contrast, in areas where the patients or carriers belong to different ethnic populations, it can be demanding and costly. In the greater Paris area, most β-thalassemia carriers requesting antenatal diagnosis are migrants from various parts of the Mediterranean Basin (mainly North Africa, but also Italy, Portugal, Spain, Greece, Turkey) or Southeast Asia. Another difficulty also arises from late presentation of at-risk couples to the diagnostic center, rendering difficult the determination of the parental β-thalassemia allele types before fetal sampling.

To overcome these problems, we have developed a laboratory procedure allow-

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ing the detection of nearly any sequence change in the human β-globin gene within one or two days. The procedure that we are now using combines specific DNA amplification and denaturing gradient gel electrophoresis (DGGE) of the PCR products. DGGE is an electrophoretic system that allows the separation of DNA fragments differing by as little as a single base change, according to their melting properties.[8,9] Prior amplification of the DNA fragments allows their detection on the ethidium bromide–stained gel, obviating the need for radioactive probes. As the globin gene is small, it is possible, using computer simulation of DNA melting, to position appropriate couples of PCR primers that generate a few overlapping amplified gene segments easily analyzed in a single DGGE run. In our hands, this procedure allows efficient screening of the different mutations altering the human β-globin gene and significantly improves antenatal diagnosis of β-thalassemia.

MATERIALS AND METHODS

Computer Simulation of DNA Melting

Melting calculations were performed on a PC-type microcomputer using the MELT and SQHTX programs provided by Dr. Leonard Lerman. These computer algorithms predict the melting behavior, including the positions and melting temperatures (T_ms) of the melting domains, of a DNA fragment on the basis of its nucleotide sequence. This information was used to determine the denaturing gradient conditions and electrophoresis time that would result in maximum gel resolution.[8]

Oligonucleotide Design and Synthesis

Oligonucleotides were synthetized on an Applied Biosystem 391A DNA synthesizer. The position of the sequences for oligonucleotide primers that would generate the DNA fragments best suited for DGGE was selected by their predicted melting behavior. The same 40-bp GC-clamp, designed as previously described,[10] was used for each set of primers.

DNA Amplification

PCR was performed using a Perkin-Elmer Cetus thermal cycler as previously described,[11] except that each primer was used at 0.1 μ*M* and 40 PCR cycles (1 min at 94°C, 1 min at 55°C, and 1 min at 72°C) were carried out. Because the resolution of the assay is increased by examining heteroduplexes, the PCR cycles were followed by a 10-min denaturation step at 94°C and a 1-h annealing step at 56°C, in order to optimize the annealing of allelic DNA strands.

Denaturing Gradient Gel Electrophoresis

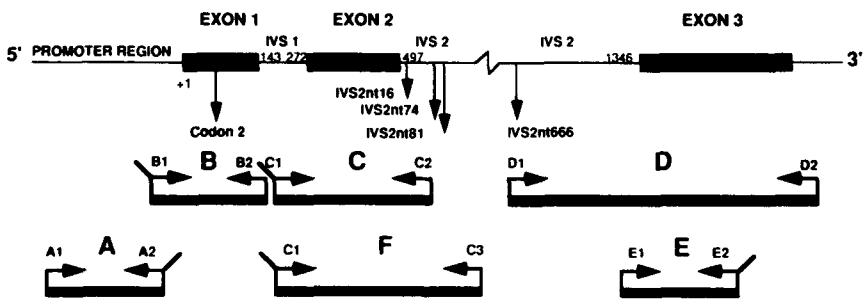
One-fifth of each sample was loaded onto a 6.5% polyacrylamide gel containing a 10–60% (fragments D and F) or 30–80% (fragments A, B, C, and E) denaturant

linear gradient parallel to the direction of electrophoresis and run at 160 V for 5 h. The gel apparatus and conditions for DGGE were exactly as previously described.[11]

RESULTS

In order to optimize the detection of single base changes in the human β-globin gene, we analyzed the melting behavior of the gene sequences using a computer algorithm. This allowed us to select positions for the oligonucleotide primers that would generate five overlapping DNA fragments best suited for DGGE. In this system, the DNA fragments move through a polyacrylamide gel containing a linear gradient of increasing DNA denaturant concentration and melt in discrete segments (melting domains), each segment melting at a distinct temperature (T_m) which is highly dependent on its nucleotide sequence. As a DNA molecule enters the concentration of denaturant where its lowest temperature melting domain melts, it forms a branched structure which has a lower mobility in the gel matrix. The migration of two DNA fragments differing by single base changes will be retarded at different positions in the gel, and consequently they will be separated at the end of the run. However, DGGE cannot be used to detect single base changes located in a DNA fragment that melts as a single domain. This problem is overcome by attaching a GC-rich segment (GC-clamp) by incorporation onto the 5′ ends of the amplified DNA sequence during PCR.

FIGURE 1 shows the position of the primers and of the gene segments analyzed. The five fragments A, B, C, D, and E are used to analyze the entire β-globin gene. A GC-clamp is attached to fragments A, B, C, and E since they melt as a single domain.



FRAMEWORK	CODON 2	IVS2 nucleotides			
		16	74	81	666
1	C	C	G	C	T
2	C	C	T	C	T
3	T	G	T	T	C
3a	T	G	T	C	C

FIGURE 1. Schematic representation of the structure of the regions of the β-globin gene used for polymerase chain reaction amplification and denaturing gradient gel electrophoresis. The nucleotides that differ between frameworks 1, 2, 3, and 3a in codon 2 and in IVS-2 are indicated at the bottom of the figure. nt, nucleotide.

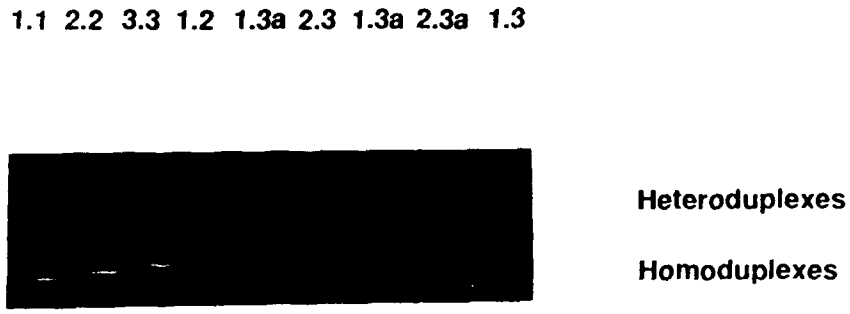


FIGURE 2. DGGE analysis of fragment F amplified from individuals homozygous or heterozygous for the different β-globin gene frameworks. The frameworks present in each sample are indicated above each lane; 1.1, homozygote for framework 1; 1.3a, heterozygote for frameworks 1 and 3a, etc.

To obtain the highest resolution, the five fragments are each less than 700 bp in length. Each melts in at least two domains; the final position of each in the gel will thus depend on the nucleotide sequence of the lowest melting domain. In the procedure we have designed, each part of the globin gene is contained at least once in the lowest melting domain of one fragment. The neutral polymorphisms occurring in the human β-globin gene could complicate interpretation of the data. These sequence variations define four frameworks: 1, 2, 3, and 3a. To take the nucleotide variations into account and to limit the number of controls during electrophoresis, the fragments were chosen to contain three polymorphisms (codon 2, C/T; IVS-2 nt 16, C/G; and IVS-2 nt 666, T/C) which are identical between frameworks 1 and 2 (C, C, T) on the one hand and 3 and 3a (T, G, C) on the other hand. In these conditions, a single control DNA from an individual heterozygote for frameworks 1 (or 2) and 3 (or 3a) allows the evaluation of the melting behavior of fragments B, C and D, whereas any normal control permits the analysis of fragments A and E. In addition, fragment F (See FIG. 1), which contains at its 3′ end three IVS-2 nucleotide polymorphisms (at positions nt 16, C/G; nt 74, G/T; and nt 81, C/T) defining the four frameworks, enables the determination of those carried by each individual analyzed using the procedure (FIG. 2). Overall, the set of gene segments shown in FIGURE 1 permits a complete analysis of the various target regions of the human β-globin gene.

The resolution of the gel system is greatly increased by the heteroduplexes that are formed during the later cycles of PCR.[10] Although they introduce a certain complexity into the pattern of bands observed (see FIG. 2), it is actually advantageous. These heteroduplexes melt early during DGGE because they are destabilized by single base mismatches. Consequently, a mutant fragment will separate from the wild type even when the homoduplex does not melt.

Selected examples of results obtained using DGGE are presented in FIGURES 3 and 4. The DGGE patterns observed from the analysis of individuals heterozygous for different mutations or polymorphisms lying in the DNA segments A, B, C, E, and D are shown on FIGURE 3. All of the nucleotide substitutions were distinguished from the normal sequence by DGGE. The results presented in FIGURE 4 concern a couple originating from Algeria who are at-risk for β-thalassemia. Each member of the couple is homozygous for β-globin gene framework 1, as shown by study of the melting behavior of fragment F (data not shown). Analysis of fragment A revealed

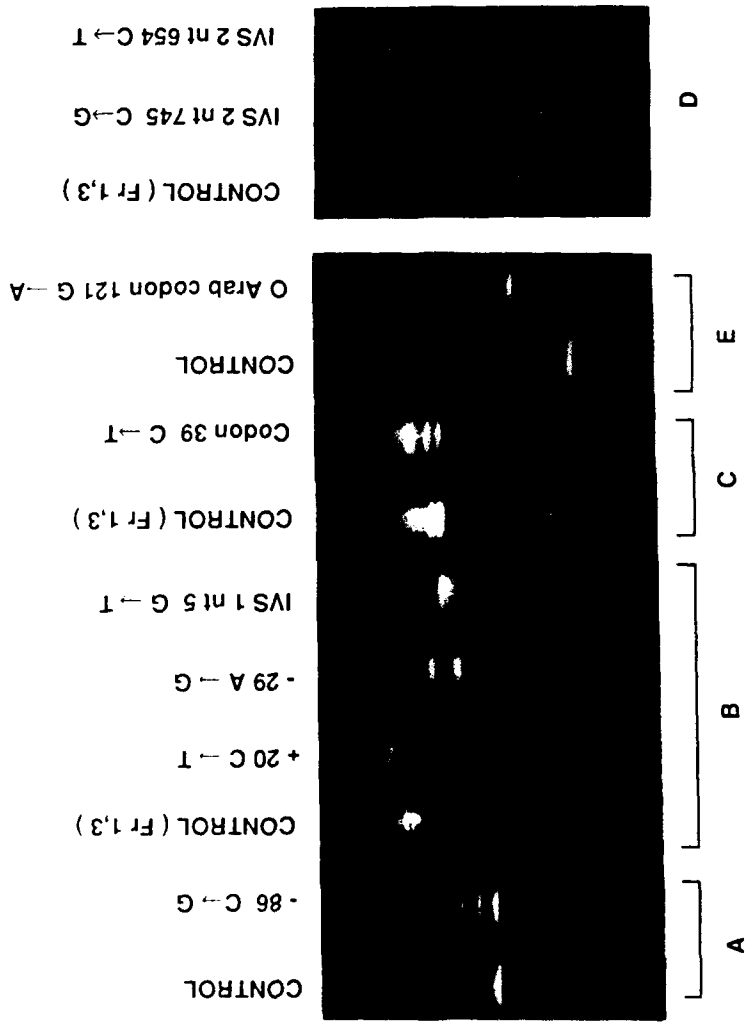


FIGURE 3. Examples of melting behaviors on DGGE analysis of mutant or polymorphic fragments from different parts of the gene in heterozygotes. **(Left panel)** Fragments A, B, C, and E were electrophoresed in a denaturing gradient gel (30–80% denaturant) for 4 h at 160 V. **(Right panel)** Fragment D was electrophoresed in a 10–60% denaturing gel (4 h, 160 V). The nature of the nucleotide changes or of the controls run in parallel is depicted on the top of each lane.

that the man is homozygous for a nucleotide substitution which changes the mobility of this fragment. DGGE of fragment B demonstrated the presence of a stabilizing nucleotide change in this individual, whereas the DGGE pattern of fragment C was abnormal in the woman. In these two cases, the four-band patterns were characteristic of the heterozygous state. Direct sequence analysis of the altered DNA fragments established that the man carries a nt −190 G-to-A nucleotide polymorphism and the frameshift 6 defect (confirmed by restriction analysis with the endonuclease *Mst* II), and that the woman has the nonsense codon 39 mutation.

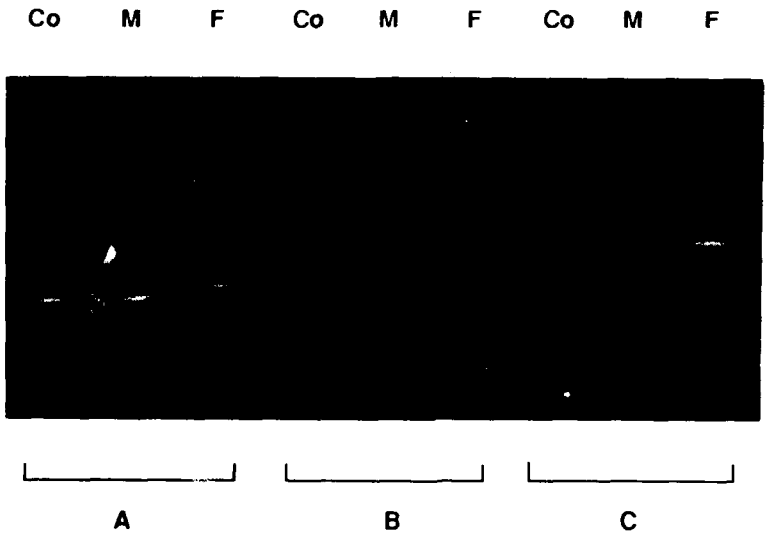


FIGURE 4. DGGE of β-globin gene DNA fragments from a couple of β-thalassemia carriers prior to antenatal diagnosis. Fragments A, B, and C were run in a 30–80% denaturing gel (4 h, 160 V). Each member of the couple is a homozygote for β-globin gene framework 1. Fragment A in the father (F) migrates more slowly than does the normal fragment A in the control (Co), which is always seen as one band since it does not contain any nucleotide sequence polymorphism, in contrast to the other fragments. In the same individual, the DGGE pattern of fragment B contains heteroduplex species. This fragment carries a mutation which causes the domain to melt at a higher temperature, producing a mutant homoduplex band running further into the gel than does the normal homoduplex. Fragment C of the mother (M) contains a mutation which causes the domain to melt at lower temperature.

CONCLUSION

The diagnostic approach described in this paper presents certain advantages, yielding answers in nearly all the cases studied and within a reasonable time. In our hands, all the mutants analyzed displayed a characteristic shift in mobility allowing unequivocal identification. In addition, it is immediately clear which part of the gene contains the defect. This greatly facilitates further characterization by direct sequencing of the relevant PCR product, the PCR primers serving as sequencing primers. We believe that the combined use of PCR, GC-clamps, and DGGE is an excellent

alternative to the use of ASO hybridization and that it will become a major method for characterizing the defects that produce other human monogenic disorders.

ACKNOWLEDGMENT

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β-Thalassemia Intermedia in Turkey

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INTRODUCTION

β-Thalassemia intermedia is not rare in Turkey.[1-4] In recent years, studies at the gene level have indicated that several mutations are associated with β-thalassemia intermedia.[5] It has been observed that some mutations, such as the IVS-1 nt 6 (T→C) mutation and mutations in the β-globin gene promoter, are mild, while for others the association of the mutation with certain haplotypes which have the C→T mutation at nt −158 5′ to the $^{G}\gamma$ globin gene may be responsible for the mildness of the condition. Changes in the α gene number were also found to influence the phenotypic expression of the disease. A comparison of some hematological data among patients with various abnormalities is presented here, together with similar results for their heterozygous parents.

MATERIALS AND METHODS

Forty-one patients with β-thalassemia intermedia belonging to 33 families were attending the hematology clinic at Hacettepe Children's Hospital, Ankara, Turkey, and, together with 45 parents, are the subjects of this study. None of the patients required regular blood transfusions. Detailed clinical and hematological data and results of family studies for some of these patients have been reported previously.[3-5] Hematological and hemoglobin composition analyses followed routine procedures.[6,7] Blood samples collected in EDTA were mailed to Augusta, Georgia (U.S.A.) for further studies. HPLC methodology was used for hemoglobin (Hb) quantitation. DNA was isolated from peripheral white blood cells; haplotyping and identification of the thalassemic mutation, as well as α-globin gene mapping, followed previously published procedures.[8-10]

RESULTS

Seventeen of the 41 patients had β^0-thalassemia; eight of these were homozygotes for the frameshift mutation (−AA) at codon 8 (FSC8), and nine were homozygotes for the G→A mutation at IVS-2 nt 1 (TABLE 1). Three patients with a homozygosity for $^{G}\gamma^{A}\gamma(\delta B)^0$-thal were found to have a 13-kb deletion (TABLE 2).

Ten of the 21 patients with β^+-thalassemia had the IVS-1 nt 6 (T→C) homozygosity (TABLE 1). Five patients were compound heterozygotes for one mild and one severe β-thalassemia determinant (TABLE 2). In the remaining patients, Hb Knossos, the C→T mutation at position −101, the T→A mutation at position −30, the G→A and G→C mutations at IVS-1 nt 5, and the G→A mutation at IVS-1 nt 110 were other β-thalassemia determinants associated with mild disease (TABLE 2). All

TABLE 1. Hematological Data for Homozygotes with Various Mutations

Mutation[a]	n[b]	Hematological Data (average ± SD)					
		Hb (g/dl)	RBC (10^{12}/1)	PCV (l/l)	MCV (fl)	Hb A_2[c] (%)	Hb F[d] (%)
IVS-1 nt 6 (T→C)	10	7.68 ± 0.72[e]	3.30 ± 0.62	0.24 ± 0.01[h]	67.9 ± 5.9[i,j]	5.60 ± 1.3[h,k]	11.0 ± 6.4[h,k]
IVS-2 nt 1 (G→A)	9	7.80 ± 1.35[f]	3.10 ± 0.34[g]	0.24 ± 0.03[g]	73.1 ± 2.2[j]	1.75 ± 0.3[k]	98.2 ± 0.3[k]
FSC8 (−AA)	8	9.68 ± 1.05[e,f]	4.00 ± 1.65[g]	0.29 ± 0.04[g,h]	73.0 ± 1.6[i]	1.40 ± 0.2[h]	98.6 ± 0.2[h]

[a]nt, nucleotide; FSC8, frameshift at codon 8.
[b]n, number of patients.
[c]By microcolumn chromatography.[7]
[d]By an alkali denaturation technique[6] and HPLC.[5]
[e]$p < 0.02$, FSC8 vs. IVS-1 nt 6.
[f]$p < 0.05$, FSC8 vs. IVS-2 nt 1.
[g]$p < 0.02$, FSC8 vs. IVS-2 nt 1.
[h]$p < 0.001$, FSC8 vs. IVS-1 nt 6.
[i]$p < 0.05$, FSC8 vs. IVS-1 nt 6.
[j]$p < 0.05$, IVS-2 nt 1 vs. IVS-1 nt 6.
[k]$p < 0.001$, IVS-2 nt 1 vs. IVS-1 nt 6.

TABLE 2. Hematological Data for Compound Heterozygotes and for Patients with a Homozygosity for Some Rare Mutations

Patient	Sex/Age (yr)	Hb (g/dl)	RBC (10^{12}/l)	PCV (l/l)	MCV (fl)	Hb A_2[a] (%)	Hb F[b] (%)	Spleen Size (cm)	α Genes	Haplotype	Mutations[c]
A.E.	M/27	8.0	3.8	0.25	77	1.8	39	(17)[d]	αα/αα	IV/I	FSC8/Hb Knossos
A.K.	M/20	7.0	4.1	0.30	71	2.0	95	6	αα/αα	III/I	IVS-2 nt 1/IVS 1 nt 110
O.S.	M/18	7.8	4.3	0.31	71	3.2	44	3	αα/αα	VII/I	IVS-1 nt 6/IVS-1 nt 110
M.O.	M/5	8.5	4.1	0.26	72	3.4	38	10	—[e]	—[e]	IVS-1 nt 1/IVS-1 nt 6
I.O.	M/15	9.3	3.9	0.26	65	2.8	47	(12)[d]	—[e]	—[e]	IVS-2 nt 1/IVS-1 nt 6
V.S.	M/8	6.4	3.3	0.24	73	2.5	62	(14)[d]	—[e]	—[e]	FSC8/IVS-1 nt 110
K.K.	M/11	9.0	3.8	0.31	81	2.2	54	8	ααα/αα	I/II	nt −101/β^{39}
Z.K.	F/12	11.3	5.2	0.38	73	4.6	34	2	αα/αα	I/II	nt −101/β^{39}
A.T.	M/9	7.5	2.1	—[e]	72	0.0	100	—[e]	αα/αα	—[e]	$\delta\beta^{13kb}/\delta\beta^{13kb}$
S.P.	M/20	10.4	5.9	—[e]	87	0.0	100	—[e]	—[e]	—[e]	$\delta\beta^{13kb}/\delta\beta^{13kb}$
I.P.	M/18	8.1	4.6	—[e]	80	0.0	100	—[e]	—[e]	—[e]	$\delta\beta^{13kb}/\delta\beta^{13kb}$
C.M.	M/15	11.0	3.6	—[e]	67	3.8	94	(10)[d]	αα/αα	IV/IV	IVS-1 nt 5 (G→C)/ IVS-1 nt 5 (G→A)
A.K.	F/8	8.6	—[e]	0.29	—[e]	3.8	92	(12)[d]	—[e]	IX/IX	IVS-1 nt 110/IVS-1 nt 110
O.K.	M/16	10.0	2.8	0.18	64	9.0	29	3	αα/αα	VII/VII	nt −30/nt −30

[a]By microcolumn chromatography.[7]
[b]By HPLC.[5]
[c]FSC8, frameshift at codon 8 (−AA); nt, nucleotide; IVS-2 nt 1 (G→A); IVS-1 nt 6 (T→C); IVS-1 nt 110 (G→A); IVS-1 nt 1 (G→A); nt −101 (C→T); β^{39}, nonsense codon 39 (C→T); $\delta\beta^{13kb}$, 13-kb deletion; nt −30 (T→A); IVS-1 nt 5 (G→C); and IVS-1 nt 5 (G→A).
[d]Splenectomy was performed; figure in parenthesis indicates the size of the spleen before splenectomy.
[e]Not studied.

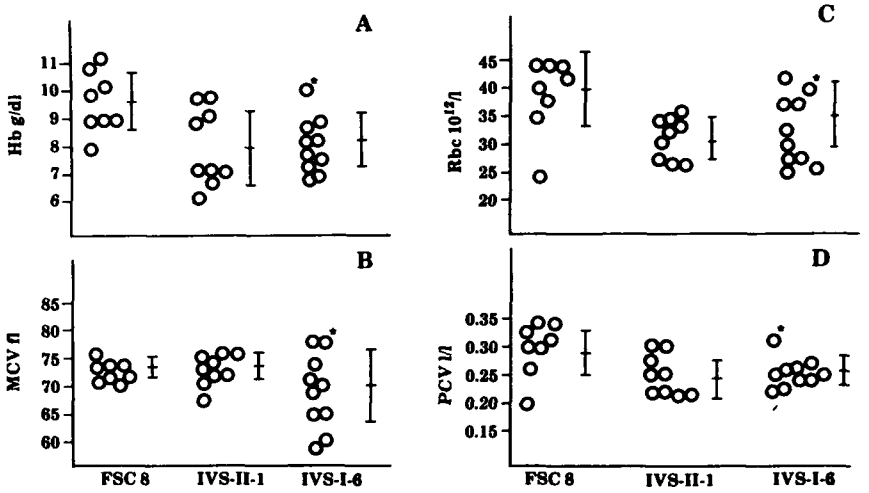

FIGURE 1. Distribution and averages ± SD of some hematological parameters in patients with the frameshift at codon 8 (FSC 8: −AA), IVS-2 nt 1 (IVS-II-1: G→A), and IVS-1 nt 6 (IVS-I-6: T→C) mutations. *Asterisk* (*) indicates patient with a coexistent α-thalassemia-2 trait (−α/αα).

patients with FSC8 and the one patient with the G→A and G→C mutations at IVS-1 nt 5 were homozygous for haplotype IV. All patients with the IVS-2 nt 1 (G→A) mutation were homozygous for haplotype III. One patient with a homozygosity for the G→A mutation at IVS-1 nt 110 was homozygous for haplotype IX. The C→T mutation at nt −158 5′ to the $^{G}\gamma$ globin gene was found in all subjects with haplotypes III, IV, and IX. The mean values for some hematological parameters are given in TABLE 1, and the distribution of some of these values is shown in FIGURE 1. The

TABLE 3. Hematological Data in Heterozygotes with Various Mutations

		Hematological Data (average ± SD)				
Mutation[a]	n[b]	Hb (g/dl)	RBC (10^{12}/l)	MCV (fl)	Hb A_2[c] (%)	Hb F[d] (%)
IVS-1 nt 6 (T→C)	15	12.2 ± 1.4	5.36 ± 0.51	69.7 ± 3.1	4.20 ± 0.85[f]	0.85 ± 0.3[f,h,j]
IVS-2 nt 1 (G→A)	12	11.8 ± 1.1	5.74 ± 0.48	67.6 ± 0.9	4.75 ± 0.95	2.75 ± 1.7[i]
FSC8 (−AA)	11	12.2 ± 1.4	5.76 ± 0.38	68.1 ± 3.8	5.40 ± 0.75[f,g]	2.50 ± 1.2[f]
IVS-1 nt 110 (G→A)[e]	23	12.0 ± 0.8	5.57 ± 0.21	68.7 ± 4.4	4.60 ± 0.65[g]	1.65 ± 1.1[h]

[a]nt, nucleotide; FSC8, frameshift at codon 8.
[b]n, number of patients.
[c]By microcolumn chromatography.[7]
[d]By alkali denaturation.[8]
[e]Heterozygotes with severe β^+-thalassemia mutation who were diagnosed at Hacettepe Children's Hospital.
[f]$p < 0.05$, FSC8 vs. IVS-1 nt 6.
[g]$p < 0.05$, FSC8 vs. IVS-1 nt 110.
[h]$p < 0.05$, IVS-1 nt 6 vs. IVS-1 nt 110.
[i]$p < 0.05$, IVS-1 nt 6 vs. IVS-2 nt 1

TABLE 4. Hematological Data for Some Parents Heterozygous with Rare Mutations

Patient	Sex/ Age (yr)	Hb (g/dl)	RBC (10^{12}/1)	PCV (l/l)	MCV (fl)	Hb A_2[a] (%)	Hb F[b] (%)	α Gene	Haplotype	Mutation[c]
Mother Ka.	F/32	14.0	—[d]	—[d]	97	3.4	1.9	αα/αα	I	nt −101 (C→T)
Mother Ko.	F/28	10.3	4.3	0.31	70	5.5	1.5	αα/αα	VII	nt −30 (T→A)
Father Ko.	M/30	15.0	5.6	0.45	81	6.1	1.1	αα/αα	VII	nt −30 (T→A)
Mother T.	F/adult	14.2	—[d]	—[d]	71	3.2	9.9	−α/αα	—[d]	$\delta\beta^{13\,kb}$
Father T.	M/adult	14.5	—[d]	—[d]	70	3.1	19.0	ααα/αα	—[d]	$\delta\beta^{13\,kb}$
Mother P.	F/adult	13.0	—[d]	—[d]	75	2.3	11.7	—[d]	—[d]	$\delta\beta^{13\,kb}$
Father P.	M/adult	12.0	—[d]	—[d]	70	2.2	10.7	—[d]	—[d]	$\delta\beta^{13\,kb}$

[a]By microcolumn chromatography.[7]
[b]By alkali denaturation.[6]
[c]nt, nucleotide; $\delta\beta^{13\,kb}$, 13-kb deletion.
[d]Not studied.

hematological data for patients with a homozygosity for some rare variants or with compound heterozygosities are given in TABLE 2.

The hematological data for the parents with heterozygosities for one of the above-listed mild thalassemia mutations are given in TABLES 3 and 4 and in FIGURES 2 and 3, while data for adults with a heterozygosity for a more severe β^+-thalassemia mutation (G→A at IVS-1 nt 110) are listed for comparison. The presence of an α-globin gene triplication was found in one of the siblings with the mutation at nt −101–nonsense codon 39 combination and in one of the $^A\gamma^G\gamma(\delta\beta)^0$-thalassemia heterozygotes (TABLES 2 and 4), while α-thalassemia-2 (−α/αα) was present in one patient with a homozygosity for the IVS-1 nt 6 (T→C) mutation and in the second adult with the $^A\gamma^G\gamma(\delta\beta)^0$-thalassemia heterozygosity (TABLE 4 and FIGS. 1 and 2).

DISCUSSION

Three mutations, namely FSC8 (−AA), IVS-2 nt 1 (G→A) and IVS-1 nt 6 (T→C), are the most common mild β-thalassemia alleles in Turkey.[5] However, six additional mild β-thalassemia mutations were found in the families we studied.[11-13] It has been suggested that patients with β^0- or β^+-thalassemia and an associated C→T mutation at nt −158 5′ to the $^G\gamma$-globin gene have a thalassemia with milder features due to an enhanced γ chain production in response to anemic stress.[14] This situation existed in the homozygotes for FSC8 (−AA) or for the IVS-2 nt 1 (G→A) mutation, in the one patient with a compound heterozygosity for the IVS-1 nt 5 G→A and G→C mutations, and in a patient with a homozygosity for the IVS-1 nt 110 (G→A)

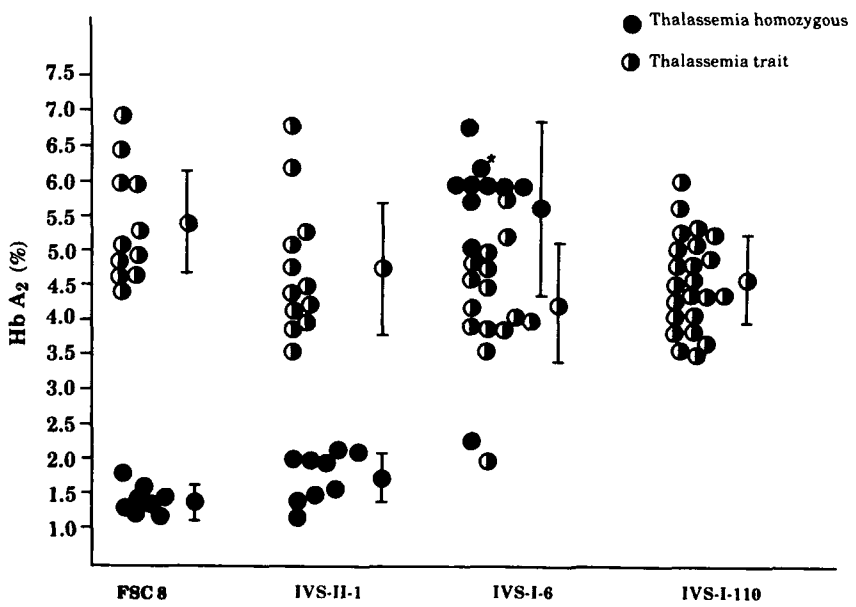

FIGURE 2. Distribution and averages ± SD of the Hb A_2 levels in heterozygotes (thalassemia trait) and homozygotes for the listed β-thalassemia mutations. *Asterisk* (*) indicates patient with a coexistent α-thalassemia-2 trait (−α/αα).

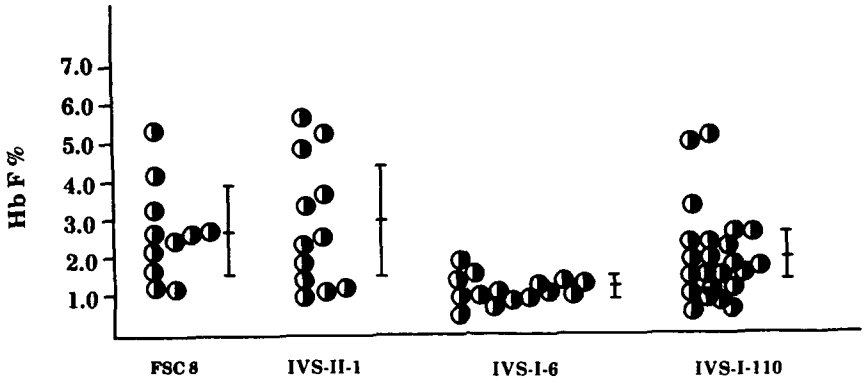

FIGURE 3. Distribution and averages ± SD of the Hb F levels in heterozygotes for the listed β-thalassemia mutations.

mutation. All these patients have a high Hb F level (TABLES 1 and 2). The G→A mutation at IVS-1 nt 110 is one of the most common β^+-thalassemia alleles in the Mediterranean Basin that is usually associated with haplotype I and severe disease.[15]

It has been suggested that changes in the number of α-globin genes cause an alteration in phenotypical expression of β-thalassemia or sickle cell anemia.[12,16,17] The Hb F level was higher in one of the two siblings with the compound heterozygosity for the mutations at nt −101 (C→T) and codon 39 (C→T) who also had an α-globin gene triplication (ααα/αα), but the higher level of Hb F was not associated with milder disease (TABLE 2). A similar observation was made in one subject with a heterozygosity for $^{G}\gamma^{A}\gamma(\delta\beta)^{0}$-thalassemia; this person had a triplication of the α-globin genes (ααα/αα) and a high Hb F level of 19% (TABLE 4). However, the one patient with a homozygosity for the T→C mutation at IVS-1 nt 6 and an α-thalassemia-2 trait (−α/αα) had the highest Hb level, in excess of 10 g/dl (FIG. 1), and a low Hb F level of 3.4%. A low Hb F level of 10% was also present in the parent with a $^{G}\gamma^{A}\gamma(\gamma\beta)^{0}$-thalassemia heterozygosity and an α-thalassemia-2 trait (−α/αα).[18]

The Hb A_2 level in the patient with a homozygosity for the nt −30 (T→A) mutation was a high 9%, suggesting that there may be an activation of the δ gene *in cis* of this mutation. It is of interest to note that the nt −101 (C→T) mutation is a silent one.[12]

Data from statistical analyses have suggested that the hematological expression of the disease is milder in patients with the FSC8 (−AA) frameshift than in patients with the IVS-2 nt 1 (G→A) or IVS-1 nt 6 (T→C) mutations ($p < 0.05$ for the differences in the mean values of Hb, RBC, and MCV: TABLE 1). As both the FSC8 (−AA) and the IVS-2 nt 1 (G→A) mutation have the C→T mutation at −158 5′ to the $^{G}\gamma$ gene, it is difficult to explain the statistically significant differences in some of the hematological parameters between these two groups. This may suggest the presence of some other factor(s) playing a role in the amelioration of the expression of the disease in the patients with FSC8 (−AA). The Hb A_2 level was very low in both groups of patients, while the Hb A_2 values in the FSC8 (−AA) heterozygotes were significantly higher than those for the other groups.

A review of some of the hematological parameters in the heterozygotes for several mild mutations and for a severe β^+-thalassemia mutation (i.e., G→A at IVS-1 nt 110) shows that a prediction of the type of β-thalassemia mutation in heterozy-

gotes is not possible by routine hematological examination (TABLE 3 and FIGS. 2 and 3).

The data presented in this study indicate that the molecular pathology and the phenotypical expression of β-thalassemia intermedia are quite heterogeneous. The results also emphasize that in addition to the several mild β-thalassemia mutations, other characteristics of the chromosome with the β-thalassemia allele and the number of α-globin genes play an important role in the pathogenesis of β-thalassemia intermedia.

SUMMARY

DNA data have been collected for 41 patients with β-thalassemia intermedia without transfusion dependency. They belonged to 33 families, and 45 of their parents were included in the study. Eight patients were homozygous for the frameshift at codon 8 (−AA), and nine were homozygous for the IVS-2 nt 1 (G→A) mutation; haplotypes IV and III, respectively, were associated with these mutations. Three patients had a $^{G}\gamma^{A}\gamma(\delta\beta)^{0}$-thalassemia homozygosity, characterized by a deletion of 13 kb. Of the remaining subjects, ten had a homozygosity for the IVS-1 nt 6 (T→C) mutation, and five were compound heterozygotes for one mild and one severe thalassemia determinant. Combinations with Hb Knossos, the T→A mutation at nt −30, the C→T mutation at nt −101, the G→A and G→C mutations at IVS-1 nt 5, and the G→A mutation at IVS-1 nt 110 were the other thalassemia determinants resulting in β-thalassemia intermedia in the six remaining patients. Haplotypes IV and IX were associated with the latter three mutations. The C→T mutation at nt −158 5′ to the $^{G}\gamma$ gene was characteristic for haplotypes III, IV, and IX.

Genotype and phenotype correlation indicated significant differences in some of the hematological parameters among patients with the frameshift at codon 8 (−AA) or with the IVS-2 nt 1 (G→A) mutation, with both the frameshift at codon 8 and the T→C mutation at IVS-1 nt 6, and with both the IVS-2 nt 1 (G→A) and IVS-1 nt 6 (T→C) mutations. Statistically significant differences were found in the mean values for hemoglobin (Hb) A_2 in heterozygotes with the frameshift at codon 8 (−AA) and the IVS-1 nt 5 (G→A) mutation. Variations in the number of α-globin genes resulted in modifications of the phenotypical expression of the β-thalassemia intermedia determinants.

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Molecular Analysis of Atypical β-Thalassemia Heterozygotes[a]

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INTRODUCTION

Heterozygous β-thalassemia is usually silent at the clinical level and manifests itself hematologically with reduced blood cell volume (mean corpuscular volume, MCV) and hemoglobin content per cell (mean corpuscular hemoglobin, MCH) and increased Hb A_2 levels.[1] However, a number of β-thalassemia heterozygotes either present clinically with thalassemia-like symptoms or show unusual hematological characteristics and are thus referred to as "atypical heterozygotes."

Under this broad definition of atypical heterozygotes, we include four different categories of heterozygous β-thalassemia, namely those with (a) normal MCH and MCV, (b) normal Hb A_2 levels (type 2 silent β-thalassemia), (c) normal red cell indices and Hb A_2 levels (type 1 silent β-thalassemia), or (d) a thalassemia-like phenotype (thalassemic hemoglobinopathies). Because of the above hematological manifestations, some of these β-thalassemia carriers may be missed by β-thalassemia carrier screening. In the last few years, we and others have characterized at the molecular level a number of these atypical heterozygotes, thus improving our diagnostic capabilities in carrier screening as well as in the diagnosis of thalassemia-like clinical manifestations. In this paper we review the current state of knowledge of the molecular pathology of these interesting groups of β-thalassemia heterozygotes.

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HETEROZYGOUS β-THALASSEMIA WITH NORMAL MCV AND MCH

β-Thalassemia heterozygotes who coinherited α-thalassemia, either in the form of two α-globin gene deletions (−α/−α) or in the non-deletion form affecting the major α2-globin gene, tend to have larger and better hemoglobinized red blood cells as compared to β-thalassemia heterozygotes with a normal complement of four α-globin structural genes. In a limited proportion of these double heterozygotes for β- and α-thalassemia, the MCV and MCH values are normal.[2,3] These heterozygotes are missed in β-thalassemia carrier screening by MCV-MCH determination. The practical implication of this finding is that for most of those carriers in populations with a high frequency of both α- and β-thalassemia, screening for β-thalassemia carriers should include Hb A_2 determination in the first set of tests.

HETEROZYGOUS β-THALASSEMIA WITH NORMAL Hb A_2 LEVELS

A number of families in which some of the β-thalassemia heterozygotes have normal Hb A_2 levels have been described. Genetic studies have produced evidence for the presence of segregating β- and δ-thalassemia mutations, most likely indicating that most of those carriers with normal Hb A_2 levels are indeed double heterozygotes for δ- and β-thalassemia.[4,5] Molecular analysis of the δ-globin genes in such families has defined, so far, several mutations which most likely silence (δ^0-thalassemia) or reduce (δ^+-thalassemia) the output of δ-globin chain from the affected δ-globin locus.

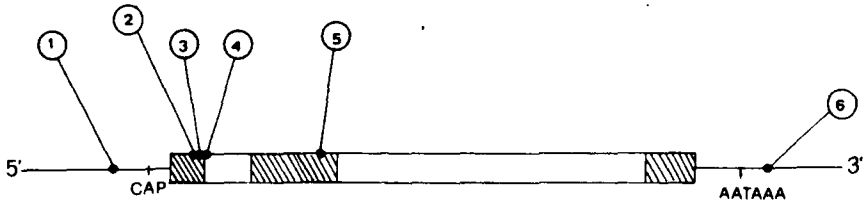

FIGURE 1. Schematic representation of the δ-globin gene. The positions of the point mutations so far described are indicated: (**1**) −77 (C→T), (**2**) codon 27 (G→T), (**3**) codon 30 (G→C), (**4**) IVS-1 nt 1 (T→C), (**5**) codon 91 (+A), (**6**) 69 bp 3′ to poly(A) (G→A).

These mutations, which are represented schematically in FIGURE 1, include a deletion in the ψβ–δ region and point mutations affecting the δ-globin gene. The single type of deletion δ-thalassemia hitherto described involves a loss of 7201 bp and has its 5′ breakpoint at a site 3′ to the ψβ-globin region and its 3′ breakpoint in the middle of IVS-2 of the δ-globin gene. This mutation, which was detected several years ago in linkage with the β^+ IVS-1 nt 5 G→A mutation and is referred to as Corfù δβ-thalassemia,[6] has been observed more recently by our group in chromosomes in which a normal β-globin gene resides.[7]

Point mutations in the δ-globin gene (FIG. 1) include a T→C substitution 77 bp 5′ to the cap site,[8] a G→T substitution at a position corresponding to amino acid 27 (δ^+27),[9] a T→C substitution at position 1 of IVS-1 (δ^0 IVS-1 nt 1 T→C),[9] a G→C substitution at the second position of codon 30 (the last nucleotide of exon 1),[10] and a

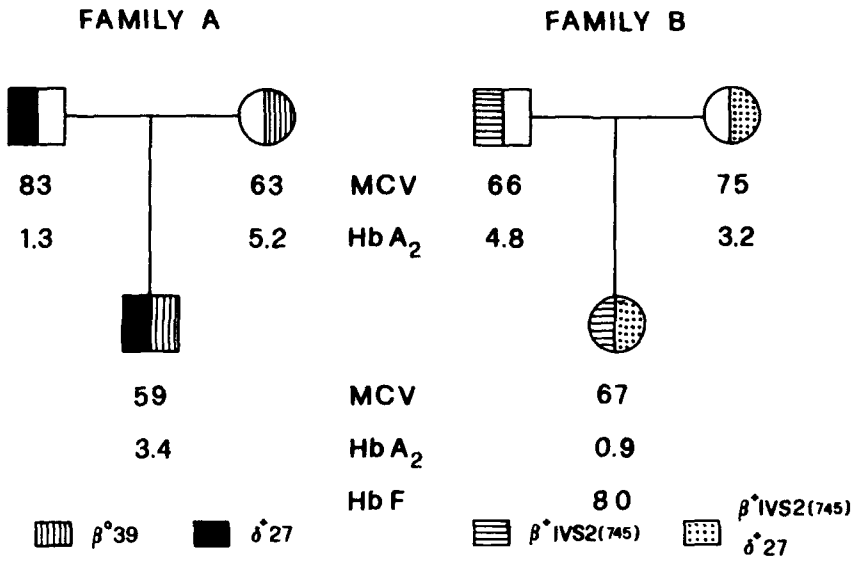

FIGURE 2. Normal Hb A_2 β-thalassemia. Pedigree of two families with δ^+27 β-thalassemia either in *cis* (Family A) or in *trans* (Family B).

frameshift (+A) in the third position of codon 91.[11] It is interesting to note that identical nucleotide substitutions in the corresponding positions of the β-globin gene, producing β-thalassemia, have been detected for three of the δ-thalassemia mutations hitherto described[10] (δ^+27, δ^0 IVS-1 nt 1, and G→C at codon 30). Identical nucleotide substitutions in the β- and δ-globin genes may arise either as independent mutations or as the result of gene conversion events. Deletion and non-deletion δ-thalassemia in *trans* to β-thalassemia have so far been described in several families. However, the δ^+27 mutation has been observed by us either in *trans* or in *cis* to heterozygous β-thalassemia,[12] as depicted in FIGURE 2.

In addition to the above well-defined δ-thalassemia mutations, we have recently observed a G→A substitution 69 bp downstream of the poly(A) addition site in the δ-globin gene from a chromosome containing the β^+ IVS-2 nt 745 mutation in three generations of a family in which this mutation was consistently associated in the heterozygous state with normal Hb A_2 levels.[10] Dot blot analysis with specific oligonucleotide probe detected the +69 (G→A) mutation in all heterozygotes for the β^+ IVS-2 nt 745 mutation of the proband family but failed to reveal this mutation either in a group of normal individuals or in unrelated heterozygotes for the β^+ IVS-2 nucleotide 745 mutation of the same Sardinian descent or of different origin. The +69 (G→A) mutation may be responsible for the low δ chain output from this determinant by affecting mRNA end formation, or it could be a silent polymorphism not affecting the function of the δ-globin gene.

A δ-thalassemia mutation most likely exists in the chromosome containing the Hb Knossos mutation in the Mediteranean population and may be responsible for the normal Hb A_2 level in otherwise typical β-thalassemia heterozygotes.

In addition to the findings of normal Hb A_2 levels with double heterozygosity for δ- and β-thalassemia, borderline-to-normal Hb A_2 levels have also been detected in β-thalassemia heterozygotes for a number of β-thalassemia mutations, including β^+ IVS-1 nt 110 and β^+ IVS-1 nt 6.

SILENT β-THALASSEMIA

Under the heading of silent β-thalassemia are included those β-thalassemia heterozygotes who have normal MCV, MCH and Hb A_2 values and are thus defined solely by an imbalance of the β/α-globin chain biosynthesis ratio (type 1 silent β-thalassemia).[1] This heterozygous β-thalassemia phenotype has hitherto been found solely in one or both parents of patients affected by those mild forms of thalassemia which are referred to as thalassemia intermedia.

At the present time, only a single silent β-thalassemia mutation, namely a C→T substitution at position −101 to the cap site, has been characterized at the molecular level. This mutation was detected in several families of Turkish or Italian descent with a proband affected by thalassemia intermedia who was a compound heterozygote for the −101 (C→T) mutation and a typical high-Hb A_2 β-thalassemia mutation[13,14] (FIG. 3). The −101 promoter mutation affects the distal CACCC box, which is one of the promoter elements of the β-globin gene. It is worthwhile to note that this mutation reduces globin output much less than do mutations affecting the proximal CACCC box (C→G at position −87 and C→T at position −88), indicating that the distal CACCC box is less crucial for the function of the β-globin gene than is the proximal one. The −101 (C→T) mutation is contained in haplotype I and is a very common cause of thalassemia intermedia in patients of southern Italian ancestry homozygous or heterozygous for this haplotype.[13]

In addition to this well-defined β-thalassemia mutation, we have recently detected in an individual of southern Italian origin with the silent β-thalassemia phenotype a complex rearrangement consisting of the deletion of a nucleotide (T) and the addition of a sequence (ATATA) at position −530 5′ to the cap site of the β-globin gene (FIG. 3).[14] A similar rearrangement, the deletion of a nucleotide (T) and the addition of an ATA sequence (−T,+ATA), was detected several years ago in the β-globin gene of a silent β-thalassemia carrier of Albanian descent.[15] More recently it was found by our group in three siblings with thalassemia intermedia.[31] They inherited from their father, a classical β-thalassemia carrier, a normal β-globin gene and the −530 promoter rearrangement, isolated or in combination with a triple α-globin gene arrangement. From their mother, who had the silent β-thalassemia carrier phenotype, they inherited the β-101 (C→T) promoter mutation. Other studies, however, revealed the −530 promoter rearrangement (−T,+ATA) in a large number of individuals, including normals and patients with β-thalassemia, Hb E or Hb S, indicating, as suggested by Wong *et al.*,[16] that this rearrangement is most likely a polymorphism not responsible for the silent form of β-thalassemia. It is worth noting, however, that the so-called normal persons investigated for the −T,+ATA promoter rearrangement have not been studied by globin chain synthesis analysis,

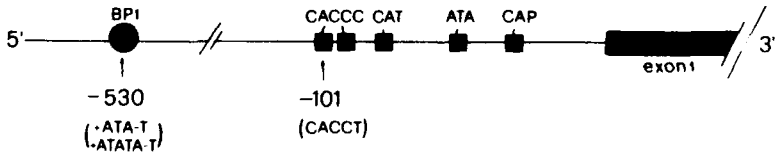

FIGURE 3. The β-globin gene promoter region. The *square symbols* represent the CAP site and the ATA, CAT, and CACCC boxes. At position −101 within the distal CACCC box, a C→T substitution is indicated (silent β-thalassemia). In the distal or expanded promoter, which runs from −100 to 600 bp 5′ to the CAP site, the *circle* indicates the binding site for BP1. Here two rearrangements, +ATA−T and +ATATA−T, have been found at position −530.

which is the only procedure allowing us to ascertain the silent β-thalassemia carrier state.

The conclusion that the −T,+ATA rearrangement is a common polymorphism is contradicted by recent studies using a DNase protection assay with nuclear extracts of K562 cells which have identified a protein designated binding protein 1 (BP1). This protein binds to DNA regions 5′ to the cap site, one of which is located between −550 and −527 bp and functions as a silencer in a transient expression assay.[17,18] BP1 binds nine times as strongly to the −T,+ATA sequence as to its normal counterpart, suggesting that the presence of this rearrangement may result in a partial suppression of the function of the β-globin gene. This may explain the silent β-thalassemia carrier phenotype presented by the original Albanian patient as well as by the individuals observed by us to carry the −T,+ATA or the similar −T,+ATATA sequence. An increased binding of BP1 to the chromosome containing the −T,+ATA rearrangement has recently been observed in SS patients of Indian descent who have a milder clinical phenotype, most likely because of a moderate reduction of the intracellular Hb S concentration.[19,20] The −T,+ATA sequence in *cis* in the Indian β^S chromosome may be responsible for the decreased expression of the β^S gene. In order to clarify the role of the −T,+ATA sequence in the expression of the in *cis* β-globin gene, we have recently screened, for the presence of this rearrangement on amplified DNA, nine normal individuals who had balanced globin chain synthesis (α:non-α = 0.9–1.15) and 14 β-thalassemia carriers. In none of them did we detect the −T,+ATA sequence, indicating that this rearrangement, at least in our population, is not a common polymorphism.[31] All these considerations raise the possibility that a reduced expression of the β-globin gene may be caused by a tighter binding of the BP1 repressor to the rearranged sequences.

Lastly, it should be noted that imbalanced α:β-globin chain synthesis in combination with normal MCV, MCH and Hb A_2 levels, namely, the silent β-thalassemia carrier phenotype, is also the pattern of phenotypic expression for the triple α-globin gene arrangement, which, by interacting with heterozygous β-thalassemia, may result in thalassemia intermedia.[21]

HETEROZYGOUS β-THALASSEMIA WITH CLINICAL MANIFESTATIONS

β-Thalassemia mutations are traditionally defined as molecular defects within the β-globin gene cluster associated with the absence of β chain production (β^0) or reduced (β^+) β chain production with, however, the β-globin chain structure unchanged. In contrast, hemoglobinopathies are defined by the presence of abnormal globins with one or more structural changes, which are synthesized at a normal rate. However, in the last few years several mutations have been observed that not only cause alterations of the globin structure, but also decrease its synthesis.[22] These are referred to as thalassemic hemoglobinopathies and are the result of a number of different molecular mechanisms, including activation of cryptic splice sites within the exon, loss of a normal termination codon, or instability of the globin chain. Among the latter group are included a number of β-thalassemia mutations which produce in the heterozygous state a thalassemia intermedia–like phenotype of varying severity. In the large majority of these thalassemic hemoglobinopathies, the hemoglobin (Hb) molecule is so unstable that it is missed by the method of Hb analysis commonly in use in clinical hematology (FIG. 4). Some of them, such as Hb Indianapolis,[23] may be identified by globin chain synthesis analysis using short-term incubation.

Within the highly unstable group of Hb variants, we and others have recently defined a subgroup which is characterized by the localization of the mutation in the

third exon of the β-globin gene (FIG. 4). These defects include frameshifts, missense and nonsense mutations.[24-28] Frameshift mutations result in elongated β-chains, while nonsense mutations are associated with truncated globin chain molecules. Some of these highly unstable variants are characterized by the presence of inclusion bodies, which result from the precipitation of the extremely unstable Hb variant together with the resulting excess of α chains (inclusion body heterozygous β-thalassemia). These β-thalassemia mutations show a dominant transmission pattern or occur sporadically.[24]

Because of their severity, it is most likely that these rare unstable variants have not come under selective pressure and hence have not reached high gene frequency in malarious populations, in contrast to the common β-thalassemia mutations.

From the practical point of view, we may conclude that the presence of abnormal, very unstable Hb should be suspected in any sporadic patient showing the clinical phenotype of thalassemia intermedia and having both parents hematologically normal or in families in which this phenotype shows a Mendelian dominantly transmitted pattern. In this context, the best method to accomplish diagnosis is the detection of the mutations by direct sequencing of the amplified β-globin gene.

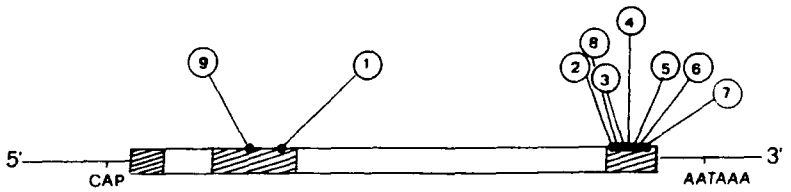

FIGURE 4. Graphic representation of the β-globin gene. Positions of the mutations responsible for the production of highly unstable Hb variants are indicated: (**1**) codon 94 (+GT = Hb Agnana);[30] (**2**) codon 109 (−G = Hb Manhattan); (**3**) codon 114 (−CT+G = Hb Geneva); (**4**) codon 121 (G→T); (**5**) codon 126 (−T = Hb Vercelli); (**6**) codon 127 (Gln→Pro = Hb Houston); (**7**) codons 128/129 (−4), codons 132/135 (−11), and codon 129 (+5); (**8**) codon 112 (Lys→Arg = Hb Indianapolis); (**9**) codon 60 (Val→Glu = Hb Cagliari).[29]

SUMMARY

This paper reviews the molecular pathology of a heterogeneous group of β-thalassemia heterozygotes which may be referred to as atypical β-thalassemia. This group includes four different categories of heterozygous β-thalassemia, which are characterized, respectively, by (1) normal MCV and MCH; (2) normal Hb A_2; (3) normal MCV, MCH, and Hb A_2 and imbalanced globin chain synthesis only or, (4) the presence of clinical manifestations. The first group is represented by a limited proportion of double heterozygotes for α- and β-thalassemia. The second group includes two categories. One category is double heterozygotes for δ- and β-thalassemia with the δ-thalassemia mutation in *cis* or in *trans* to β-thalassemia. A number of δ-thalassemia mutations which produce this phenotype by interacting with β-thalassemia have been described. The other category within the second group is heterozygotes for some mild β^+-thalassemia mutations. Within the third group, conclusive evidence for a mutation within the β-globin gene cluster producing the silent β-thalassemia phenotype has been obtained solely for a C→T substitution at −101 within the CACCC box of the β-globin gene. Possible candidates are the

complex rearrangements (−T,+ATA; −T,+ATATA) found at position −530 from the cap site. In the group of thalassemic hemoglobinopathies, a series of mutations mostly located in the third exon and producing elongated or truncated molecules have been recently reported. Most of the mutations are silent at the protein level, produce inclusion bodies in peripheral erythrocytes, and show a dominant transmission pattern or occur sporadically.

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Molecular Studies of β-Thalassemia in Israel

Mutational Analysis and Expression Studies[a]

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Advances in molecular genetics have contributed greatly to the understanding of genetic disorders and their distribution in various parts of the world. Techniques of molecular cloning and sequencing led to the identification of numerous mutations which impair β-globin gene function.[1-2] The development of *in vitro* techniques of gene amplification based on the polymerase chain reaction (PCR)[3] greatly facilitated identification of point mutations by allowing rapid analysis of a large number of samples using allele-specific oligonucleotide probes. Unknown mutant alleles could then be sequenced directly following PCR amplification.[4]

Over the past several years we have applied these modern techniques to the study of β-thalassemic alleles in Israel. Being the Holy Land for three major religions and a homeland for ancient cultures, Israel has attracted migrations of varied populations and as such, has an ethnic diversity probably unparalleled for a country of its size. Furthermore, the unique identities and cultural integrity of a significant portion of the population are still preserved, even after centuries of coexistence in a small geographic region. Consanguinity has been and still is practiced extensively in many communities,[5] increasing the frequency of affected offspring with recessive genetic disorders.

There are several hundred patients with β-thalassemia in Israel. Affected children continue to be born in spite of a prenatal diagnosis program initiated fifteen years ago, based on globin chain synthesis ratios in fetal blood samples. Most of the patients belong to distinct and well-defined ethnic groups. Carriership within the various ethnic groups ranges from less than 1% to as high as 20%.[6] We subdivided the groups with a high incidence of thalassemia according to geographic origin and religious background, because intermarriage between the various religious groups is rare. Accordingly, some groups with a notable incidence of β-thalassemia are Jews of

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Kurdish extraction (whose carrier frequency is about 20%),[6] Jews of Mediterranean origin (mainly from Turkey, Greece, and Morocco), Moslem Arabs, Christian Arabs, Druze, and Samaritans.[6-7] In the Arab communities, thalassemia is widespread in regions that were infested by malaria until the beginning of this century: the coastal region, the Jezreel Valley, the Hula Valley, and the Jordan Valley. There is also a very high incidence in the Gaza region.[8]

We report here some of the initial findings that emerged from the study of β-thalassemia in our population. We will describe the broad spectrum of clinical presentations, the diversity of molecular lesions at the DNA level, and the findings obtained from expression studies of a novel point mutation in the polyadenylation signal.

METHODS

Patients

A total of 66 families was studied. The patients were referred through hematology clinics throughout the country. Alternatively, families were referred by genetics clinics where they had sought genetic counseling. The composition of the families studied is as follows.

1. Jews of Kurdish extraction. Sixty two patients from 41 sibships (either Kurdish or half Kurdish), residing in all parts of Israel, were analyzed. This represents nearly all known patients of this ethnic group (62 of 64). Thirty-three families have thalassemia major and eight, thalassemia intermedia. Two intermedia patients were each found to have one parent who is a silent carrier.
2. Christian Arabs. One family residing in the lower Galilee was studied. The members of this family have a thalassemia intermedia phenotype. The mother is a silent carrier.
3. Moslem Arabs. Seventeen families from all parts of Israel were investigated. All patients except for one have thalassemia major.
4. Druze. Six families (thalassemia major) from two villages in the Galilee were analyzed.
5. Jews of other Mediterranean origin. Five families in which one or both parents were of Moroccan or other Mediterranean origin were studied. Four have thalassemia major and one has thalassemia intermedia.

DNA Analysis

DNA was prepared according to standard procedures as previously described.[9] Haplotype analysis was performed as described by Orkin *et al.*,[10] for the following restriction enzyme sites: *Hin*c II 5′ to ε, *Hin*d III sites in the $^{A}\gamma$ and $^{G}\gamma$ genes, *Hin*c II sites at the ψβ locus, *Ava* II in the β IVS-2 and *Bam*H I 3′ to the β-globin gene. The nomenclature used was as described by Orkin *et al.*[10]

PCR was performed using 1 μg of genomic DNA and primers spanning the complete β-globin gene, 1832 bp, from 166 nucleotides (nt) upstream of the cap site to 60 nt downstream from the polyadenylation site, with the following primers: 5′ primer, CCAACTCCTAAGCCAGTGCC; 3′ primer, CACTGACCTCCCACATTCCC.

Samples were analyzed on agarose gels to verify amplification (FIG. 1) and then screened using radiolabelled allele-specific oligonucleotide probes.[11] Direct genomic sequencing was performed on unknown alleles as previously described.[3]

For families in which each parent was of a different ethnic background (such as Kurdish/Moroccan), each parent's mutations were identified.

RNA Analysis

RNA was isolated from peripheral blood normoblasts of patients as previously described.[12] The patients studied carried a novel point mutation (AATAAA→AATAAG) in the polyadenylation signal. All were compound heterozygotes for the polyadenylation mutation and a frameshift mutation in codon 44 which

FIGURE 1. Ethidium bromide–stained agarose gel showing amplification products of a typical PCR reaction. (**Lane 1**) 557-nt amplification product of the 5′ end of the β-globin gene, using the following primers: 5′ primer, CCAACTCCTAAGCCAGTGCC; 3′ primer, CACCGAGCACTTTCTTGCCA. (**Lanes 2–5**) 1832-bp amplification product using primers which spanned the entire β-globin gene. 5′ primer, same as above; 3′ primer, CACTGACCTCCCACATTCCC. Markers (M) are bacteriophage φX174 *Hae* III fragments (**first lane**) and phage λ *Hin*d III fragments (**last lane**).

has been shown to encode for an unstable RNA with a very short half-life.[13] Control RNA from a non-thalassemic individual with reticulocytosis and from a patient homozygous for the frameshift-44 mutation were analyzed in parallel.

RNase mapping was performed as previously described by Melton *et al.*[14] The

following probes were utilized: exon 1 of the α-globin gene (*Hae* II–*Ava* I), exon 1 of the β-globin gene (*Bal* I–*Hae* III), exons 1–2 of the β-globin gene (*Bal* I–*Bam*H I), IVS-2 of the β-globin gene (*Bam*H I–*Eco*R I), and exon 3 of the β-globin gene (*Eco*R I–*Pst* I).

Northern blotting was performed using 1-μg quantities of normoblast RNA glyoxylated and run as previously described,[15] except that in most cases SP6-generated RNA probes were utilized. Hybridization was at 55°C for RNA probes and 42°C for the DNA probe. The final wash was performed using 0.2× SSC/0.1% SDS at 65°C for 30 min. A DNA probe was prepared from the *Dra* I fragment located 3′ to the human β-globin gene just beyond the normal polyadenylation signal. The amount of normoblast RNA used was adjusted according to the intensity of the α-globin RNA signal on densitometry tracings, and equivalent amounts were run for the β-globin probes.

RESULTS AND DISCUSSION

Clinical Presentation

Most of the families studied (57 out of 66) have thalassemia major. Nine have thalassemia intermedia. In the Kurdish Jewish population, 20% (8 of 41) of the families had an intermedia phenotype. This figure represents the true relative incidence of the two syndromes in this ethnic group, as virtually all known patients were studied. The small number of intermedia patients found among Arabs and Druze in this study may not be representative of the true incidence in these populations, since the mechanism of referral of families for study was biased toward those more severely affected. Although several hundred β-thalassemia major patients are believed to reside in Israel (nearly a hundred in the Gaza region alone), there are no accurate statistics on the prevalence of the disease in its milder forms.

Most of the patients with thalassemia major are transfused every three to four weeks and thus require chelation treatment with subcutaneous desferrioxamine. There are occasional patients with thalassemia intermedia who are rarely transfused but nonetheless have significant iron overload requiring chelation.

Three of the families with thalassemia intermedia (two Kurdish Jewish, one Christian Arab) have one parent who is a silent carrier. One of these, a Kurdish patient, was found to carry the −101 C→T mutation, previously noted to be associated with this phenotype.[16] The other two are compound heterozygotes for a β^0 allele (frameshift 44 or IVS-1 nt 1 G→A) and unknown alleles which eluded identification despite extensive sequence analyses of the β-globin gene and its immediate flanking region. The molecular basis for these presumably mild thalassemic alleles is at present obscure.

Mutational and Haplotype Analysis

Thalassemic chromosomes were analyzed for β-globin gene mutation and for Mediterranean haplotype using the molecular techniques described above. Haplotype analysis showed that Mediterranean haplotype I is the most common chromosome for mutant alleles (67% of mutant alleles were on this haplotype). Haplotypes VII, VI, IX, V and II were also represented, but at much lower frequency.

A summary of mutant alleles and their haplotypic background is presented in

TABLE 1. A total of fifteen thalassemic alleles were found in the 66 families studied (FIG. 2). Thirteen mutations were seen in Kurdish Jews and six among the other groups studied (Mediterranean Jews, Arabs, Druze). There were six mutations that to date have not been observed outside Israel.

The results of molecular analysis of β-thalassemia mutations in our country is revealing, not only in the variety of mutant alleles but also in their relative distribution in the various ethnic groups studied. It is noteworthy that although five of the mutations were shared among the various ethnic groups studied, the majority were not.

The Kurdish Jews were found to have exceptional heterogeneity of mutations, thirteen in all, with five mutations unique to this ethnic group, an astonishingly high incidence in such a small population, who were numbered at 23,000 in 1961.[5] This is consistent with their history of centuries of ethnic and geographic isolation in Kurdistan, a primarily Moslem region. Some of their mutations seem to have evolved locally within Kurdistan, mainly on the common haplotypic background of Mediterranean haplotype I. Genetic admixture of Mediterranean mutations was observed as well.

The Moslem Arabs of northern and central Israel have a smaller number of mutations, nearly all of which are prevalent in the Mediterranean region. These common mutations are linked to the haplotypes previously reported for them,[4] underscoring the likelihood that these mutations arrived by migration. The Arabs of these communities therefore reflect primarily the effects of genetic admixture from

TABLE 1. Analysis of the Mutations in Israeli β-Thalassemia Chromosomes

Mutation[a]	Alleles *n*	Alleles %	Ethnic Group	Haplotype
Frameshift 44*	24	19.5	Kurdish Jews	I
IVS-1 nt 110	22	18	Kurdish Jews	I
			Moslem Arabs	I
− 28 A→C*	18	15	Kurdish Jews	I
Nonsense 39	15	12	Kurdish Jews	VII
			Mediterranean Jews	I
			Moslem Arabs	I, II
IVS-2 nt 1 (G→A)	13	13.5	Druze	I
			Kurdish Jews	V
			Moslem Arabs	III
Poly(A) nt 6 (A→G)*	8	6.5	Kurdish Jews	VII
IVS-1 nt 1 (G→A)	5	4	Moslem Arabs	V
			Christian Arabs	Not done
IVS-1 nt 6	3	2.5	Kurdish Jews	VI
			Moslem Arabs	VI
Poly(A) deletion*	2	1.5	Moslem Arabs	Variant[b]
− 88 C→A*	1	<1	Kurdish Jews	IX
Frameshift 36–37*	2	1.5	Kurdish Jews	I
IVS-2 nt 745 (C→G)	1	<1	Kurdish Jews	VII
IVS-1 nt −1 (G→C)	1	<1	Kurdish Jews	VII
−101 C→T	1	<1	Kurdish Jews	IX
IVS-1 nt 5 (G→C)	1	<1	Kurdish Jews	VII
Unknown	5	4		
Total	122			

[a]*Asterisk* (*) denotes mutation unique to Israeli ethnic groups.
[b]− + − − + + +

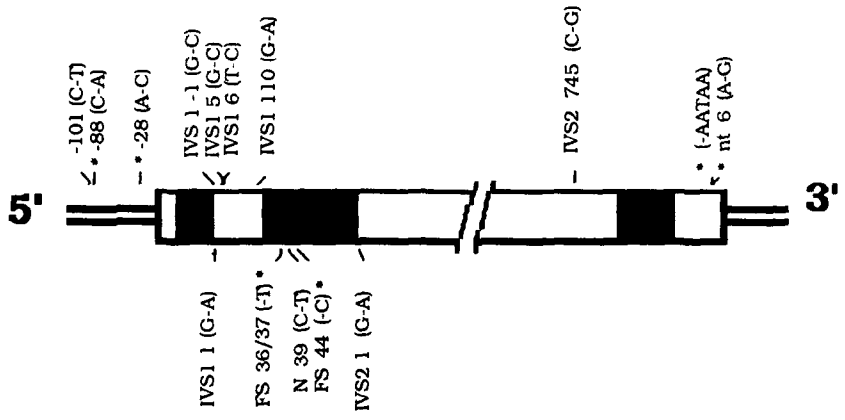

FIGURE 2. Diagram of the human β-globin gene and the fifteen β-thalassemic mutations found to date in Israel. β^+ mutations are indicated *above* the gene and β^0 mutations are indicated *below*. **Asterisks** (*) indicate mutations that have not been observed outside Israel. FS, frameshift; N, nonsense.

surrounding areas. Historically, the Islamic Empire extended from Persia (Iran) to the Atlantic Ocean during the 7th century, affording opportunities for genetic admixture to occur. These acquired mutations may then have been propagated locally, presumably due to selective pressures exerted by malaria, which was prevalent in those parts of Israel until the 1920s.

The one Arab family who was found to have the novel polyadenylation deletion mutation resides in Gaza. Studies on thalassemia are just beginning in that region, the population of which may prove to be genetically different from the Arabs of northern and central Israel.

The Druze seem to be an example of a distinct, closed ethnic group. To date, only one mutation has been found in six families of this community.

Of interest is a comparison of haplotypic backgrounds for the various alleles in the different ethnic groups studied. The IVS-1 nt 110 (G→A) mutation was seen on Mediterranean haplotype I in all ethnic groups studied. In contrast, the nonsense mutation at codon 39 was found on haplotypes I and II in Arabs, on haplotype I in Mediterranean Jews, and on haplotype VII in Kurdish Jews. Haplotype VII carries a different β-globin gene framework (a series of polymorphic base changes discovered by sequence analysis)[4] than do haplotypes I and II. The β-globin gene frameworks are believed to antecede the divergence of the races and haplotypes. Therefore, it is likely that the codon 39 nonsense mutations in Israeli patients represent recurrent mutational events. Similarly, IVS-2 nt 1 (G→A) is linked to haplotypes I in Druze, III in Arabs, and V in Kurdish Jews. Haplotype I carries a different β-globin gene framework than do haplotypes III and V, suggesting that an independent mutational event is responsible for the mutation in the Druze as opposed to that in Arabs and Jews.

Expression Studies of a Novel Mutant Allele

Functional studies on mutant alleles have contributed much information on the importance of critical nucleotide sequences to RNA processing. The finding of a

novel mutation in the cleavage-polyadenylation signal, AATAAA→AATAAG, has allowed a unique opportunity to study the effects of malfunction of RNA cleavage on gene expression.

No homozygotes for this mutation were identified. However, when this mutation is present in compound heterozygosity with a β^0 mutation (such as a frameshift or nonsense mutation), it results in β^+-thalassemia.

To clarify the mechanism whereby this mutation impairs gene function, expression studies were performed on peripheral blood normoblast RNA isolated from several patients who are compound heterozygotes for this novel mutation and for a frameshift mutation in codon 44. Northern blotting and RNase mapping experiments showed that some normal-size message is produced, about 15%–40% of the normal level (data not shown), compatible with the clinical picture of a moderate to severe β^+-thalassemia. The frameshift allele did not contribute any mature RNA, as demonstrated by control RNA isolated from a patient homozygous for that mutation, in agreement with previous studies on this allele.[13] The polyadenylation mutant alleles produced, in addition, several extended transcripts, approximately 1500, 1650, 2450, and 2900 nucleotides long. Only the shortest of these transcripts, which presumably extends to the next polyadenylation signal, about 900 nucleotides downstream of the β-globin gene, has been previously described.[17] The other three transcripts are also elongated RNA species, as revealed by hybridization to a probe specific for the region 3′ to the normal polyadenylation signal (1087-bp *Dra* I fragment), which did not hybridize to the normal message. The RNase mapping experiments also showed that the 5′ start sites and IVS-1 and IVS-2 splicing were normal. These transcripts are being evaluated further. We are also studying transcription products from another novel mutant allele, a 5-bp deletion in the polyadenylation signal, found in an Arab family from Gaza. The results of these ongoing studies will be reported in the future.

The Kurdish polyadenylation point mutation AATAAA→AATAAG has a significant negative impact on gene function, in that only 15%–40% of the usual level of normal-size β-globin mRNA can be produced in the presence of this mutation. The clinical symptoms correlate with this condition of moderate-to-severe impairment of β-globin synthesis, in that most patients with this mutation require ongoing transfusion therapy. Further work on the precise endpoints of the elongated transcripts seen in these patients will clarify the role of accessory signals for cleavage, which have been reported to be required for proper 3′ end processing.[18,19]

Application to Prenatal Diagnosis

Finally, the information derived from these studies is not only of great interest from a scientific, genetic and evolutionary point of view, but also has been applied to the field of prenatal diagnosis. Analysis of fetal tissue for the various point mutations in our population using the technique of PCR has enabled the implementation of first-trimester prenatal diagnosis. Further work should allow for a more complete understanding of this complex disease, as well as for promoting prevention of this serious genetic disorder.

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Fate of α-Hemoglobin Chains and Erythrocyte Defects in β-Thalassemia[a]

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The homozygous state of β-thalassemia is an inherited anemia related to decreased or absent synthesis of the β chain of adult hemoglobin A ($\alpha_2\beta_2$). Since β-thalassemia major is a severe condition requiring regular blood transfusions, it is difficult to obtain thalassemic red cells for study from patients with this condition. A milder form, β-thalassemia intermedia, which usually does not necessitate transfusion, has been used to describe the many morphological, rheological, and biochemical defects of thalassemic red blood cells (see Refs. 1 and 2 for review). The cause of these abnormalities is the presence of free α chains, unpaired with β chains, which are unstable and oxidize. They bind to membranes and induce oxidation of lipids and proteins and also lead to molecular cross-linking.[3-5]

In the present paper, we address the question of the relationship between the size of the soluble and insoluble α chain pools present in cells and the membrane protein defects in thalassemic erythrocytes. For this purpose we have devised methods to evaluate the pools of α chains and the thiol groups of various membrane proteins.

The soluble α chain pool present in all cells, from bone marrow cells to dense blood cells, was assessed by using a specific probe, β^A hemoglobin chains labeled with tritiated *N*-ethyl maleimide ([^{3}H]NEM); the β chains combine with the soluble α chains present in cell lysates to form ^{3}H-labeled hemoglobin and are separated and evaluated by electrophoresis.

The insoluble α chains, which remain in red cell ghosts after cell lysis and extensive washing of the ghosts, were evaluated by using polyacrylamide gel electrophoresis in the presence of urea and Triton X-100 (UT-PAGE) in order to separate simultaneously the various globin chains, α, β, $^G\gamma$ and $^A\gamma$, and the membrane proteins as previously described.[6] The standard SDS–polyacrylamide gel electrophoresis (SDS-PAGE) method was also used.[7]

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The thiol groups of the various membrane proteins were characterized by assay with dithiobisnitrobenzoic acid (DTNB) and by the binding of [^{3}H]NEM to the red cell ghost prior to UT-PAGE and autoradiography.[6]

These methods have been used to compare in human and mouse β-thalassemias and in human erythrocytes loaded with α-hemoglobin chains the fate of α chains and the expression of membrane protein defects.

HUMAN β-THALASSEMIA

Soluble α Chain Pool

We devised an affinity method to evaluate the soluble α chain pool present in all types of erythroid cells. The soluble α chain pool evaluated by this affinity method was a quantitative measure of the amount of free and soluble α chains present in intact cells *in vivo* at the time of sampling. The method is specific, very sensitive, and suitable to evaluate α chains in the presence of proteins other than hemoglobin. In addition, the sample processing is fast and performed at 0°C to prevent the loss of soluble α chains. Short term (3 h) blood storage at 4°C or freezing in liquid nitrogen has no effect on the soluble α chains.

TABLE 1. Fate of α-Hemoglobin Chains in Blood Cells

	Human β-Thalassemia	Mouse β-Thalassemia
Soluble α chains (% Hb)	0.26–1.3	< 0.01
Precipitation $t_{1/2}$	> 12 h	10 min
Proteolysis $t_{1/2}$	8 h	Not detectable
Insoluble α chains (% membrane proteins)	18.1 ± 9	35.7 ± 10

In normal individuals, free α chains are present in red blood cells but only in very small amounts, 0.067% ± 0.017 of the hemoglobin. In β-thalassemia trait, the α chain pool was only slightly increased, in spite of the unequal synthesis of globin chains (α:β ratio = 2).[8–10] This demonstrates a very efficient removal of soluble α chains in heterozygous β-thalassemia, more by proteolysis[10–12] than by precipitation, as indicated by the absence of significant amounts of insoluble α chains associated with membrane ghosts. However, the selective removal of abnormal cells containing precipitated α chains could also explain the disappearance of unpaired α chains during maturation of reticulocytes and red cell aging in heterozygous β-thalassemia.

In β-thalassemia major, traces of soluble α chains able to combine with β chains to form Hb A have been detected.[13–15] The size of the pool of soluble α chains observed in the present study (0.26–1.30%; TABLE 1) was highly variable between patients. The amount of soluble α chains was modest with respect to the total amount of hemoglobin and to the imbalance of globin chain synthesis because of the instability of α chains,[12,15] proteolysis[11,12,16–19] (see TABLE 1), and the removal of damaged cells by the reticuloendothelial system. In this regard, four splenectomized patients with relatively severe β-thalassemia, as determined by blood hemoglobin level, had the highest levels of soluble α chain (0.80–1.30%). In contrast, the two

splenectomized patients with the lowest levels of soluble α chain (0.38–0.48%) had a very mild form of β-thalassemia, suggesting that the size of the soluble α chain pool could be related to the clinical severity of the disease in splenectomized and untransfused patients.

In β-thalassemia intermedia, the soluble α chains are not restricted to reticulocytes, as suggested by the distribution of the α chain pool, which ranged from 2.4% in light cell fractions containing 32% reticulocytes to 0.3% in dense cells containing virtually no reticulocytes (i.e., <0.5%).

In bone marrow cells, a very high proportion of α chains exists in the soluble form (10% of Hb, i.e. 20% of the α chains present in Hb). This high α chain pool in the bone marrow cells of a patient with β-thalassemia intermedia contrasted with the small α chain content in normal bone marrow cells (which is two- to threefold that observed in peripheral blood). The higher level of soluble α chains in normal bone marrow cells is probably removed during erythroid maturation. The much lower soluble α chain pool in reticulocytes (2.4%) compared to that in bone marrow cells (10%) of the patient with β-thalassemia intermedia is related to the precipitation and proteolysis of α chains and differential cell death during erythroblast maturation.

Insoluble Hemoglobin Chains

Previous studies[3,20,21] of red cell ghosts from β-thalassemic patients revealed an increase in their globin content. Results obtained in the present study using UT-PAGE showed a peculiar globin chain pattern in β-thalassemia syndromes. In heterozygous β-thalassemia, the amounts of β- and α-globin chains present in ghosts were increased in similar proportion, suggesting that insoluble hemoglobin is increased and that most of the free α chains resulting from the unequal globin chain synthesis are removed or degraded. This was not due to simple contamination with soluble hemoglobin, because extensive washing of ghosts did not remove additional hemoglobin. These results probably reflect an abnormal binding of hemoglobin to membrane in heterozygous β-thalassemia.[22]

In homozygous β-thalassemia, insoluble α-hemoglobin chains have been found to be present in cell ghosts.[3,21] In the present study, ultrasonification of cell ghosts and subsequent centrifugation did not lead to separation of globin chains or hemoglobin from membrane, suggesting that hemoglobin chains were bound to membrane and not present as free inclusion bodies trapped in cell ghosts after hemolysis.[23] In addition, we showed that non–α-globin chains (β or β + γ) were also present in ghosts and that their amount increased with cell density from 5.5% of total membrane proteins in light cells to 10.8% in dense cells. In contrast, the insoluble α chains decreased from 44.2% to 24% with cell density and the α:non–α ratio of globin present in ghosts dropped from 8 to 2.2. The increase in β and γ chains in membrane ghosts in cells with increasing density indicates a reduced solubility of hemoglobin tetramers, which may be related to increased oxidation in the thalassemic cells.

Membrane Proteins

In β-thalassemia intermedia changes in membrane proteins were amplified in the lightest cells, which had a low hemoglobin content and a high amount of insoluble α chains. Both SDS-PAGE and UT-PAGE showed a reduction of spectrin (FIG. 1) and an increase of uncharacterized fractions. The decrease in spectrin is correlated with the increase in insoluble α chains (FIG. 2). Band 3 was also reduced, as shown by

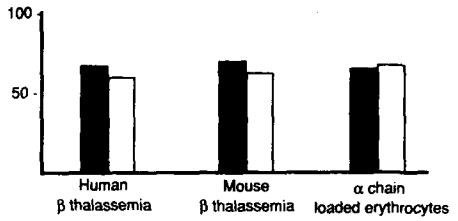

FIGURE 1. Decrease of spectrin in erythrocyte ghosts. Shown is the percentage of the normal value for the protein (■) and free thiol groups (□) in human β-thalassemia, mouse β-thalassemia, and α-hemoglobin chain–loaded normal erythrocytes.

SDS-PAGE, but to a lesser extent than were spectrin chains. Consequently, the ratio spectrin:band 3 decreased slightly in β-thalassemia. A slight deficit of spectrin has also been shown by Shinar *et al.*[21] As shown by Platt and Falcone,[24] decreased spectrin also occurs in other cells containing unstable hemoglobin (Hb Köln) and is thought to result from increased oxidation. It is well known that oxidation of spectrin will increase the proportion of high molecular weight complexes of spectrin[25,26] and impair the assembly of cytoskeleton.[27,28] Diamide at very low concentration (3 μ*M*) prevents the formation of the spectrin-4.1 complex in the presence of actin. Erythrocytes with inclusion bodies due to unstable hemoglobins have a lower content of spectrin and ankyrin in their membrane, an increase of the proportion of spectrin monomers, a decreased binding of spectrin to protein 4.1 and actin, and an alteration of ankyrin structure that decreases the binding of normal spectrin to inside-out vesicles of cells containing Heinz bodies.[29,30] Shinar *et al.* have recently shown[21] that in β-thalassemia intermedia spectrin degradation products were not detectable in cell ghosts by Western immunoblotting of SDS-PAGE, despite the use of a comprehensive array of antiproteolytic agents. The decrease in membrane spectrin is not specific to β-thalassemia or to anemia due to unstable hemoglobin, since it is also

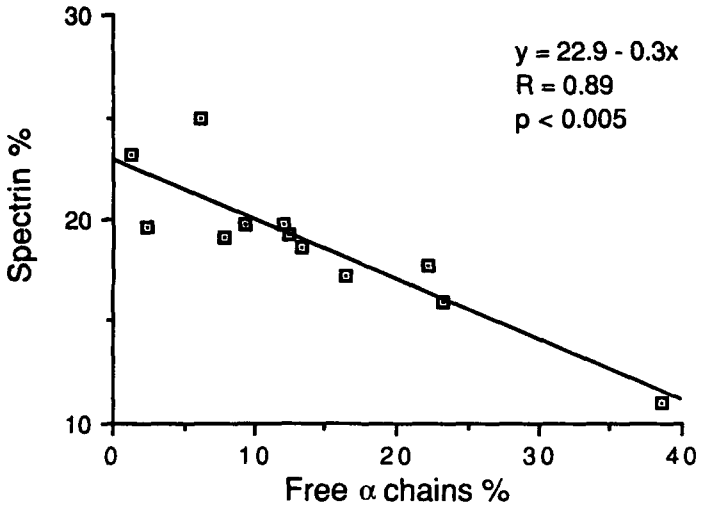

FIGURE 2. Correlation between spectrin and α-hemoglobin chains present in human erythrocyte ghosts from patients with β-thalassemia intermedia. The values shown are percentages of total membrane protein as determined by densitometry following urea-Triton polyacrylamide gel electrophoresis (UT-PAGE)[6] and staining with Coomassie blue.

observed in hereditary spherocytosis,[31] acquired disorders[32] and during blood aging *in vitro*,[33] but not during *in vivo* aging of red cells, as measured after their fractionation by centrifugation.[34] The decrease in the amount of ankyrin (34%; FIG. 3) in β-thalassemic cell ghosts was shown by UT-PAGE, which separated ankyrin from the spectrin β chain better than did SDS-PAGE.[6] The loss of ankyrin is similar to that of spectrin and should be compared to the disappearance of ankyrin from the skeleton of cells containing unstable hemoglobins, in which the ankyrin-spectrin association is altered, probably by oxidation.[24]

Some proteins are present in increased amounts in β-thalassemic cell ghosts. As suggested by Shinar *et al.*,[21] the increased presence of these polypeptides could result from attachment of cytoplasmic proteins, including globin chains or polymers of native or degraded hemoglobin chains,[23] or cross-linked membrane proteins which give rise to higher molecular weight proteins,[5] some of which are cleaved by reducing agents. Binding of globin to the membrane skeleton has been shown during aging of normal red cells *in vivo*[35,36] and in oxidation of normal red cells *in vitro*.[37,38]

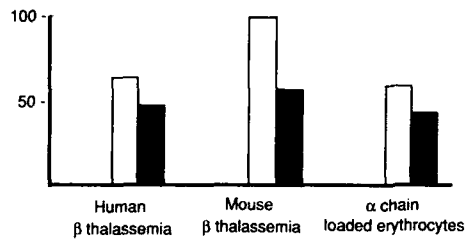

FIGURE 3. Decrease of ankyrin in erythrocyte ghosts. Shown is the percentage of the normal value for the protein (■) and free thiol groups (□) in human β-thalassemia, mouse β-thalassemia, and α-hemoglobin chain–loaded normal erythrocytes.

Topology of Sulfhydryl Groups

We confirmed that in non-transfused patients with β-thalassemia intermedia the free thiol groups of membrane proteins are decreased by 27%.[39,40] Under the conditions used, 80% of normal membrane thiol groups are reactive with NEM.[41] The decrease of thiol groups in β-thalassemic cells is not evenly distributed among membrane proteins. It affects primarily the most reactive thiol groups of spectrin (decreased by 32%; FIG. 1) and ankyrin (decreased by 65%; FIG. 3), in comparison to normal cells in which ankyrin contains a high proportion of the most reactive thiol groups in the membrane. Thiol groups exhibiting low reactivity are also decreased in spectrin and ankyrin. Other unidentified protein fractions have modified reactivity of their thiol groups.

MOUSE β-THALASSEMIA

In 1981, Johnson and Lewis described a β-thalassemia syndrome occurring in a DBA/2J mouse. This syndrome resulted from the absence of the β^{major} globin chain[42] due to a 3.7-kb deletion in the β^{major}-globin gene.[43] In the homozygous state this mutation induced anemia, high reticulocytosis and inclusion bodies in erythrocytes, conditions similar to those seen in human β-thalassemia intermedia; whereas the heterozygous state was asymptomatic.

Normally, the β^{major} gene, located at the 3′ end of the β-globin gene cluster, provides 80% of the β-globin chains. The β^{minor} gene, located 3′ of the β^{major} gene, is

responsible for the β^{minor} chain, which accounts for 20% of the β-hemoglobin chains in the normal diffuse *Hbb* genotype (Hbb^d/Hbb^d).

In the homozygous state of mouse β-thalassemia (Hbb^{thal}/Hbb^{thal}), the absence of the β^{major} chain, due to the β^{major} gene deletion, is partially corrected for by an increase in the level of β^{minor}-globin chain synthesis. The β^{minor}:α-globin chain ratio increases from 0.2 in the normal mouse to 0.7–0.8 in the homozygous β-thalassemic mouse.[44,45] This relative increase in β^{minor}-globin chain synthesis was shown to occur at the translational level, suggesting that the β^{minor} mRNA is preferentially translated in comparison to the α-globin mRNA.[45] In addition, the β:α ratio of globin chain synthesis in β-thalassemic mice can be normalized upon the use of 5-azacytidine,[46] which increases the fetal γ chain expression in human β-thalassemia.[47]

To determine to what extent mouse β-thalassemia can be used as a model for the human disease, we have investigated why a small deficiency in β-globin chain synthesis, lower than that observed in the asymptomatic heterozygous state in humans, is associated with a relatively severe clinical expression in the mouse. This study showed why in spite of a smaller β-globin chain deficiency in mouse β-thalassemia relative to human thalassemia, the disease is relatively severe and is a good model for human β-thalassemia intermedia.

Soluble α Chain Pool

Homozygous mouse β-thalassemia and human β-thalassemia intermedia have several differences of particular interest. The pool of soluble α chains is much smaller in mouse than in human β-thalassemia. While it accounted for 0.2–1.3% of hemoglobin in human β-thalassemia intermedia,[6] a pool of soluble and free α chains is not detectable in mouse red cells by the assay using a specific probe ([^{3}H]NEM-labeled β chains) which, when added to red cell lysates, is able to combine with the free and soluble α chains. Furthermore, a pool of soluble α chain in reticulocytes is barely detectable in mouse β-thalassemia when free α chains are labeled by the incorporation of a radioactive amino acid during protein synthesis. This latter method is known to be very sensitive. The radioactivity of the soluble α chain pool was only 25–30% of the total amount of hemoglobin plus α chains. When the labeling data were extrapolated to zero time, a half-life of 10 min was found. This is in contrast to human β-thalassemic cells, in which the radioactivity present in soluble α chains, which was more than 80% of the total protein (result not shown), exhibited a half-life of 8 h (TABLE 1); this was even longer in the absence of proteolysis.[12] In the mouse, the small pool of soluble α chains reflected, first, the small disequilibrium of globin chain synthesis in mouse β-thalassemia due to the increased translation of the mouse β^{minor} mRNA[4] and, second, the instability of the newly synthesized α chains, which became associated with the membrane, explaining the high proportion of insoluble α chains associated with membrane ghosts.

In addition, another difference between the α chains of human and mouse β-thalassemia was proteolysis of newly synthesized α chains; this was active in humans and absent in the mouse. The combination of these differences in the fate of α chains explains why insoluble α-hemoglobin chains are present in mouse cells in concentrations similar to those observed in human β-thalassemia intermedia.

Membrane Protein Defects

The electrophoretic profile of membrane proteins (FIG. 4a) is very similar in human and mouse β-thalassemia with regards to the decrease in spectrin (−25%;

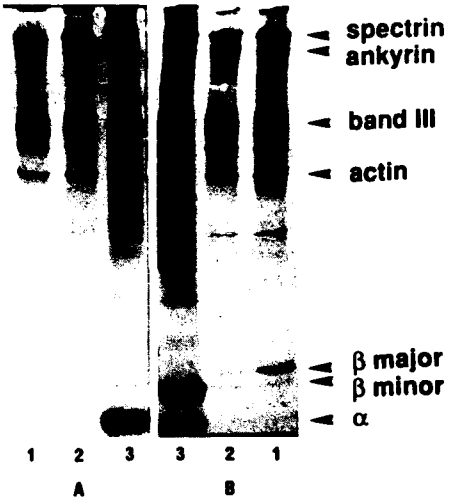

FIGURE 4. UT-PAGE electrophoresis of erythrocyte ghosts from normal mice (**lanes 1**) and mice heterozygous (**lanes 2**) or homozygous (**lanes 3**) for β-thalassemia. (**A**) Proteins stained with Coomasie blue and (**B**) following autoradiography to show the topology of the reactive thiol groups, which have bound [^{3}H]NEM.

FIG. 1) and the appearance of new protein fractions in the low molecular weight range. Additionally, the decrease in the most reactive thiol groups (−34%), which is a major feature in human β-thalassemia,[6] is also observed in mouse β-thalassemia and affects, as in humans, primarily ankyrin and spectrin (−49% and −35% respectively: FIGS 1, 3 and 4b). These results are very similar to those obtained in human β-thalassemia, with a decrease in the most reactive thiol groups of 27%, particularly of ankyrin and spectrin (−65% and −32%).

ENTRAPMENT OF PURIFIED α CHAINS AS A MODEL OF β-THALASSEMIA

As discussed in the preceding sections, excess α chains are associated with membrane defects in the β-thalassemic erythrocyte. However, it has not previously been possible to examine the process of *in vivo* development of these membrane defects in patients or in animal models since the defects were already completely developed by the time the cells were available to study. To determine directly what effects α chains have on normal membrane proteins and to follow the rate at which these changes occur, purified α chains were entrapped within normal erythrocytes by reversible osmotic lysis.[48] As has been shown previously, osmotic lysis and resealing result in resealed erythrocytes exhibiting normal morphology, hemoglobin concentration, volume, ATP concentrations, oxidant sensitivity, and deformability while allowing for the efficient entrapment of exogenous compounds.[49]

The α-globin subunit of normal purified hemoglobin was prepared by dissociation of carboxylated Hb A in the presence of parahydroxymercuribenzoate followed by ion exchange chromatography to isolate the purified carboxylated α chains.[6] The purified α chains were then entrapped within normal erythrocytes by the osmotic lysis and resealing method of Scott *et al.*[48,49] Briefly, a small volume (2–5 ml) of washed erythrocytes (80–85% hematocrit) were mixed with purified α chains and sealed in dialysis tubing (M_r cutoff of 3500). The samples were dialyzed against 1 l of lysis buffer (5 m*M* potassium phosphate buffer, pH 7.4, and 2 m*M* EDTA) for 60 min at 4°C. The tubing was then transferred to 1 l of resealing buffer (5 m*M* potassium

phosphate resealing buffer, pH 7.4, 160 m*M* NaCl, and 5 m*M* glucose) and mixed for 30 min at 37°C. Following resealing, the cells were washed with saline until the supernatant was clear.

The normal, the control-resealed, and the α chain–loaded erythrocytes were suspended to a 5% hematocrit in NCTC 109 buffer (Sigma Chemical, St. Louis, MO, USA) supplemented with 5 m*M* glucose and incubated in a shaking water bath at 37°C for up to 20 h. Aliquots were removed at the indicated times for analysis of membrane proteins, reactive thiol groups, and cellular deformability. Alterations in membrane proteins and reactive thiol groups (i.e., reduced thiols) were determined as previously described,[6] and the relative protein and reactive thiol group concentrations were determined by densitometry. The protein and reactive thiol concentrations are expressed as percentages of the total membrane protein (membrane-specific and skeletal proteins) excluding globin. The α chain concentration is expressed as a percentage of membrane-specific protein (i.e., excluding globin). Changes in cellular deformability were assessed using a Cell Transit Analyser (ABX, Levallois, France),[50-51] which determines the transit time, in milliseconds (ms), of 2000 individual cells. In addition, the Cell Transit Analyser determines the mean cell transit time for the population as well as the transit times for the 25th, 50th, 75th, 90th, and 95th percentiles of the cell population.

As previously demonstrated, osmotic lysis and resealing alone[48,49] had no substantial effects on erythrocyte structure and function but resulted in the efficient entrapment of α chains. Erythrocytes resealed in the presence of 10 mg of α chains per milliliter of packed erythrocytes had intraerythrocytic α chains concentrations of 3.8 ± 0.5% of the total hemoglobin, as determined by use of [^{3}H]NEM-labeled α chains. Analysis of membrane proteins and reactive thiol groups immediately following resealing demonstrated that they were unchanged in the control-resealed and α chain–loaded erythrocytes. (TABLE 2).

The α chain pool, while initially soluble, progressively precipitated with incubation at 37°C, and a membrane adherent fraction became apparent. The amount of membrane-bound α chains in the α chain–loaded cells increased from less than 3% initially to 48% of the normal membrane protein after 20 h of incubation at 37°C (FIG. 5). Interestingly, the rate of membrane adherence closely parallels the rate at which oxygenated α chains autoxidize to methemoglobin (FIG. 5). The membrane-associated α chains arose entirely from the entrapped pool, since no β chain band was detected by electrophoresis to indicate dissociation of normal hemoglobin. The normal and control-resealed erythrocytes exhibited no α chain bands after 20 h of incubation (FIG. 5).

TABLE 2. Initial Membrane Protein and Reactive Thiol Groups of Normal, Control-Resealed, and α Chain–loaded Erythrocytes As Analyzed by UT-PAGE

Sample	Spectrin	Ankyrin	Band 3	Actin
Protein concentration[a]				
Normal	33.9 ± 5.5	5.8 ± 2.0	45.2 ± 3.3	3.8 ± 0.6
Resealed	35.4 ± 2.8	6.0 ± 2.2	44.4 ± 3.4	3.8 ± 0.3
α Chain	37.2 ± 3.8	7.5 ± 2.6	40.0 ± 4.6	2.9 ± 0.4
Reactive thiol groups[a]				
Normal	19.2 ± 4.2	26.9 ± 2.3	25.2 ± 7.6	16.4 ± 8.7
Resealed	19.4 ± 1.6	26.3 ± 0.9	23.6 ± 5.8	15.2 ± 6.6
α Chain	21.6 ± 2.5	30.4 ± 2.5	23.3 ± 4.1	11.8 ± 4.5

[a]Values expressed as percentage of total membrane protein excluding globin; $n = 3$.

Entrapment of α chains had significant effects on membrane proteins and reactive thiol groups. The α chain–loaded erythrocytes exhibited a progressive and severe loss of the spectrin and ankyrin protein bands, which was associated with the membrane adhesion of the entrapped α chains. Following 20 h of incubation, spectrin and ankyrin concentrations were decreased by 28.8% and 36.5%, respectively, from their initial values (FIGS. 1 and 3). The concentrations of other membrane proteins (e.g., Band 3) were unaffected by α chain loading. No significant alterations in membrane proteins were observed in the normal and control-resealed erythrocytes during the course of the experiments. Preceding or concurrent with the loss of membrane proteins, a significant decrease in protein-associated reactive thiol groups was also observed in the α chain–loaded cells. Following 20 h of incubation, spectrin- and ankyrin-associated reactive thiol groups in the α chain–loaded cells were decreased by 28.0% and 44.5% of their initial values, respectively (FIGS. 1 and 3). The reactive thiol groups of the normal and control-resealed cells were unchanged during the course of the incubation. As shown in FIGURES 1 and 3, the observed changes in the spectrin and ankyrin protein concentrations and reactive thiol groups are very similar to those seen in both human and mouse β-thalassemia.

Coincident with the observed membrane protein and thiol changes, the α chain–loaded erythrocytes exhibited a significant loss of cellular deformability, a functional abnormality which is also characteristic of β-thalassemia.[52] Incubation of the α chain–loaded erythrocytes at 37°C resulted in a progressive increase in the mean cell transit time and in the maximum transit times required for the 25th, 50th, 75th, 90th, and 95th percentiles of cells to pass through the micropore filter (FIG. 6). As shown, at 0 h, 90% of the α chain–loaded cells require less than 1.9 ms to pass

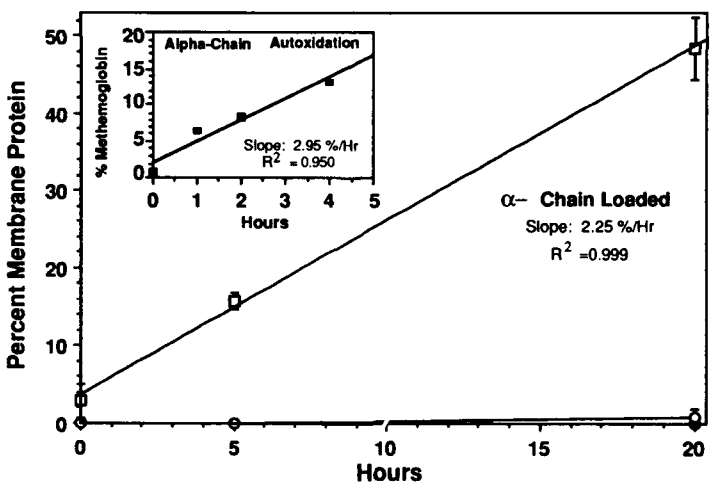

FIGURE 5. Membrane-adherent α chains increased rapidly over time in the α chain–loaded erythrocytes (□) following incubation at 37°C. As shown in the *insert*, the rate of α chain membrane deposition closely paralleled the rate of α chain autoxidation (■). Conversely, normal (●) and control-resealed (○) erythrocytes exhibited no substantial membrane-associated α chains. α Chain concentration was determined by UT-PAGE and is expressed as the percentage of membrane-specific proteins (excluding globin). The relative protein concentrations were determined by densitometry and are expressed as the mean ± SD at 0, 5, and 20 h incubation (n = 3).

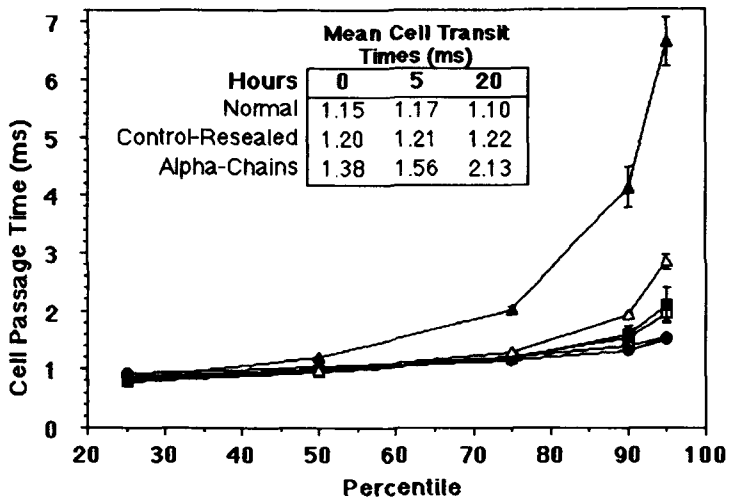

FIGURE 6. Erythrocyte deformability is significantly altered as a consequence of entrapment of α chains. Shown is the mean time (±SD), in milliseconds, required for the 25th, 50th, 75th, 90th, and 95th percentile of the cells (>2000 cells per sample) to pass through a micropore filter of defined characteristics. **Initial samples** (0 h): normal (○), control-resealed (□), and α chain loaded (△). **Final samples** (20 h of incubation): normal (●), control-resealed (■), and α chain loaded (▲). The mean cell transit times of the entire sample populations at 0, 5, and 20 h of incubation are shown in the *insert*.

though the membrane pore. Conversely, at 20 h, 90% of the α chain–loaded cells can pass through the filter in less than 4.1 ms. Normal and control-resealed erythrocytes exhibited no substantial losses in cellular deformability following incubation for 20 h. The loss of cellular deformability closely paralleled the changes in membrane proteins, loss of reactive thiol groups, and the membrane adherence of α chains.

In summary, entrapment of purified α chains within normal erythrocytes resulted in decreases in membrane proteins and reactive thiol groups in a pattern similar to that observed *in vivo* in human and mouse β-thalassemia.[6] Furthermore, entrapment of α chains resulted in loss of cellular deformability; such loss is a characteristic feature of β-thalassemic erythrocytes.[52] The results of this study also demonstrate that the protein and thiol changes and the decreased cellular deformability characteristic of β-thalassemic cells occur very rapidly in the presence of soluble α chains. This model for the β-thalassemic erythrocyte provides a tool by which the fate of the excess α chains (precipitation, membrane binding, and proteolysis), as well as their pathophysiological effects (cellular oxidation and loss of cellular deformability), can be examined. Additionally, this model may be useful in the evaluation of possible therapeutic approaches which might be capable of preventing or minimizing the erythrocyte abnormalities observed in β-thalassemia.

CONCLUSIONS

The α-hemoglobin chain pools and the cellular defects in human β-thalassemia and two model systems (mouse β-thalassemia and normal erythrocytes loaded with

α-hemoglobin chains) were examined. These studies allowed for the determination of the fate of α chains and the membrane defects characteristic of β-thalassemia. A direct correlation between the increase in insoluble α-hemoglobin chains and decreases in the membrane-associated protein concentration and reactive thiol groups of spectrin and ankyrin was observed. These changes were further associated with a loss of cellular deformability in the α-hemoglobin chain–loaded erythrocytes. These studies demonstrate the major roles played by proteolysis and precipitation of α-hemoglobin chains in modulating disease expression at the erythrocyte level.

SUMMARY

The fate of α-hemoglobin chains and the cause of membrane protein defects in thalassemic erythrocytes have been studied in: (1) human β-thalassemia syndromes, (2) mouse β-thalassemia, and (3) normal human erythrocytes loaded with purified α-hemoglobin chains. The similarity and differences observed in these three systems underline the importance of insoluble α chains and the direct relationship between the amount of these chains and the membrane protein defects. Indeed, in addition to the α/non-α ratio of globin chain synthesis, the proteolysis and instability of α chains are major factors in modulating the cellular defects.

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Differences in the Pathophysiology of Hemolysis of α- and β-Thalassemic Red Blood Cells

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INTRODUCTION

The severe forms of the thalassemic syndrome are characterized by hemolysis, in addition to the ineffective erythropoiesis which results from impaired globin chain synthesis.[1] The major abnormalities within red blood cells (RBC) of thalassemic patients result from the precipitation of the complementary, non-thalassemic globin chain: α chain in β-thalassemia and β chain in α-thalassemia.[2] During denaturation and precipitation of the hemoglobin subunits, activated oxygen radicals are produced, which may enhance and further damage the different components of the thalassemic RBC[3] by a mechanism similar to that suggested for other hemoglobinopathies.[4] In addition, iron, which is present in excess in thalassemic RBC in the form of ferritin and hemosiderin, might be particularly harmful as a catalyst in the formation of the free oxygen radicals.[5]

Alterations in different cellular and membrane properties have been detected in thalassemic RBC.[6] They consist of abnormalities in the lipid bilayer, including alterations in phospholipid and cholesterol content, evidence for increased lipid peroxidation, altered intracellular cation content, and abnormal distribution of sialic acid residues.[6] These changes might contribute to abnormal clustering of transmembrane proteins and exposure of new antigens, which in turn could result in binding of autologous antibodies.[6,7] All of the above effects may lead to the premature destruction of thalassemic RBC and their removal from the circulation by the reticuloendothelial system.[6]

Current studies suggest that there are distinct differences between the pathophysiology of α- and of β-thalassemia. They may result from the differences in the physicochemical properties of the unpaired globin chains present in excess in each of the clinical entities of the disease.[8,9] Free α-globin chains are unable to form a stable tetramer; therefore, they precipitate as inclusion bodies early in the RBC life span, prior to release of the erythroblasts from the bone marrow.[10] β-Globin chains in excess assemble into relatively stable tetramers also known as Hb H.[11] These

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tetramers are less stable than are the tetramers of normal hemoglobin A ($\alpha_2\beta_2$) and will eventually precipitate and form inclusion bodies about halfway through the RBC life span.[12] These differences in the stability of the two major globin chains within the RBC might be responsible for the differences in morphology, mechanical properties, and membrane protein functions depicted in α- and β-thalassemia. In this communication we shall review recent data on the different patterns of RBC pathology in the severe form of α-thalassemia (Hb H disease) and the homozygous form of β-thalassemia (thalassemia major and intermedia). These alterations may account for the different clinical severity of the two major forms of the disease.

MORPHOLOGICAL CHANGES

The unique morphological appearance of thalassemic RBC in the severe forms of the disease reflects a summation of alterations in different RBC components, which may contribute to the premature hemolysis of these RBC. These changes are more evident in RBC of splenectomized patients, where the numerous pathological cells are not removed by the spleen and thus can be identified in the peripheral blood. Under light microscopy there are no major differences in the morphology of RBC from the two forms of thalassemia, with the appearance of microcytosis, target cells, deformed RBC, and basophilic stippling.[13] However, when ultrastructural studies were performed by electron microscopy, different patterns of inclusion body formation and precipitation were depicted in the two forms of the disease.

In homozygous β-thalassemia, studies on splenectomized patients revealed the presence of intranuclear and intracellular inclusion bodies in erythroblasts, as well as in mature erythrocytes.[14] These inclusion bodies were mostly found in the central and peripheral portions of the RBC cytoplasm; they had indistinct edges and tended to become confluent and to fuse with each other.[15] No marginated forms of inclusion bodies were found to be attached to the cell membrane, and no intimate contact between the inclusion bodies and the membrane was detected.[14,15] In addition, myelin membrane forms and alterations in the nucleus and nuclear membranes were found, particularly in RBC with abundant inclusion bodies (FIG. 1).[14]

In Hb H disease (the severe form of α-thalassemia), both the rate and locations of inclusion body formation were different from those seen in β-thalassemia. Some of the excess β chains rapidly precipitated to form intraerythroblastic inclusions[16] that could be detected as intracytoplasmic, but not intranuclear, inclusions, unlike those present in β-thalassemic RBC. In addition, they were well defined and showed clear-cut edges.[16] However, most of the inclusion bodies were membrane associated, specifically, with the inner surface of the membrane, causing its protrusions (FIG. 1).[17,18] These inclusion bodies looked very similar to those formed by incubation of normal RBC with phenylhydrazine.[19] It has been suggested that the mechanism for the close association between the inclusion bodies and the membrane involves formation of mixed disulfide bonds between residual reactive thiol groups in the precipitated β-globin chains and thiol groups of membrane proteins,[20] similar to the mechanism for Heinz body formation in unstable hemoglobinopathies.[21]

It should be noted that the formation of inclusion bodies within RBC of patients with α-thalassemia depended on the severity of the genetic mutation.[22] In addition, the presence of other genetic abnormalities, such as the Hb Constant Spring or Hb Mahidol genotypes, in patients with α-thalassemia may also modify the morphological appearance of the precipitated β chains.[22,23]

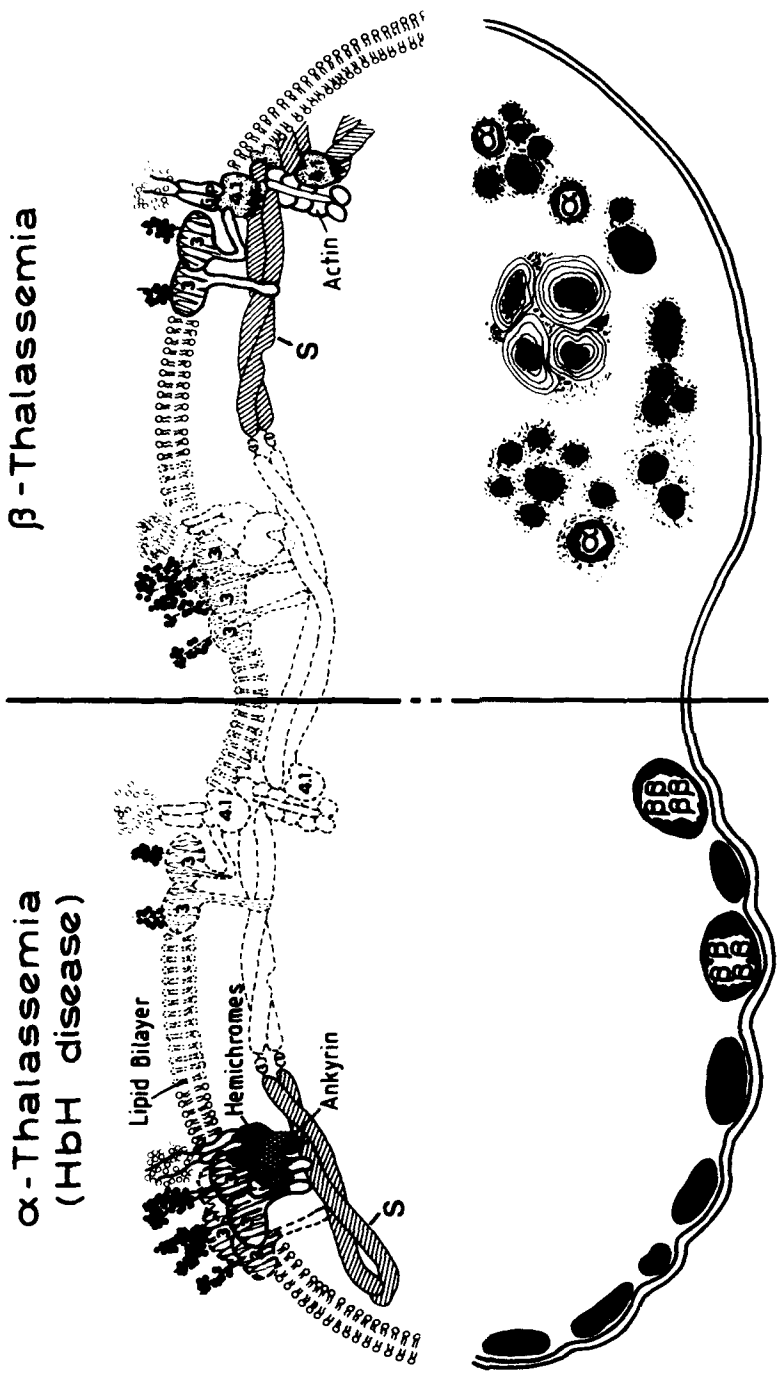

FIGURE 1. Schematic representation of RBC in α- and β-thalassemia. The upper part of the sketch depicts membrane structures, including the lipid bilayer, integral membrane protein 3 (3), glycophorins (GP), ankyrin, and the skeletal proteins spectrin (S), actin, and 4.1 (4.1). While in α-thalassemia there are abnormalities seen in assays of the interaction of spectrin with inside-out vesicles (**left panel**), in β-thalassemia the abnormal interaction is between spectrin, actin, and 4.1 (**right panel**). The lower part of the sketch depicts the presence of excess globin chains and the pattern of inclusion body (IB) precipitation. In α-thalassemia, there are β-chain tetramers, and the IBs are membrane associated; while in β-thalassemia, precipitates of α-globin chains are detected, and the IBs are intracytoplasmic and intranuclear, accompanied by myelin figures.

Protein synthesis in bone-marrow cells of patients with α- and β-thalassemia also differed considerably. High-resolution autoradiographic studies of erythroblasts in β-th lassemia showed that the majority of the cells containing moderate-to-large quantities of inclusion bodies failed to mature due to marked depression of protein synthesis.[23] This observation could explain in part why many of the abnormal very young RBC were already phagocytized by bone-marrow macrophages.[23] Autoradiographs of α-thalassemic RBC showed evidence for continued active protein synthesis in all early and late erythroblasts, including those with intracytoplasmic inclusion bodies.[23] These findings are consistent with a study showing that new β-globin chains were added to the existing inclusion bodies in α-thalassemic RBC, as these RBC were aging within the circulation.[12] It is also in agreement with previous ferrokinetic studies which suggested that there was much less ineffective erythropoiesis in Hb H disease than in homozygous β-thalassemia.[24]

CHANGES IN MECHANICAL PROPERTIES

The ability to undergo marked deformation during passage through the microvasculature of the spleen is crucial to the RBC for performing their function of oxygen delivery and determines their life span.[25] This property is influenced by membrane deformability, cell surface-to-volume ratio, and cytoplasmic viscosity.[26] Abnormal deformability of β-thalassemic RBC was first reported in 1979 by Tillman and Schroter.[27]

A recent study aimed at evaluating the rheological properties of α- and β-thalassemic RBC showed that cellular and membrane deformability was altered not only by the increased globin content per se, but also by the extent and type of chains interacting with the membrane.[28] As a result of globin precipitation, both α- and β-thalassemic RBC had an increased ratio of surface area to volume, increased membrane rigidity, and decreased ability to undergo cellular deformation. However, in this same study, it was shown that there were differences in membrane mechanical stability and the state of cell hydration between the two major forms of thalassemia. While the mechanical stability of membranes from α-thalassemic RBC was only marginally elevated, in β-thalassemic RBC it was substantially decreased. As a result, the latter RBC fragmented twice as easily when compared to α-thalassemic or control RBC. Cellular dehydration, reflecting deranged membrane transport function, was also found only in β-, not in α-, thalassemic RBC.

As the deformability and mechanical stability of RBC are regulated by interactions of their skeletal and integral membrane proteins, some of the above-mentioned alterations may result from the abnormal skeletal protein interactions described in thalassemia,[29] which differ in the two major forms of the disease (see below).

CHANGES IN MEMBRANE STRUCTURE AND FUNCTION

The irregular morphology of thalassemic RBC, their abnormal cation and lipid content, the altered distribution of glycoprotein and sialic acid residues,[6] and the rheological alterations described above, suggest that the RBC membranes and skeletal proteins must be severely damaged.

Electrophoretic analyses of membrane proteins of thalassemic ghosts prepared by hypotonic lysis show essentially normal polypeptide pattern in both α- and β-thalassemia.[29,30] There was an increase in the globin content of thalassemic RBC

membranes, constituting up to 8–10% of the total membrane protein content, as measured by densitometry or pyridine elution of the gels.[29,30] In addition, an increase of cytoplasmic proteins, such as catalase, was detected which has been described also in other hemolytic anemias.[31]

No quantitative differences were observed in the main components of the cytoskeleton such as spectrin, actin, and protein 4.1. However, when the various functions of cytoskeletal proteins were studied in both forms of thalassemia, qualitative, specific, and different abnormalities have been detected.[29] In α-thalassemia, there is an altered interaction between normal spectrin and its membrane binding sites on the thalassemic inside-out vesicles (IOVs) (FIG. 1). The IOVs were defective and bound only half the amount of spectrin bound by control or β-thalassemic IOVs. In order to confirm the latter observation, normal RBC membranes were incubated with isolated α- or β-globin chains.[32] Only IOVs derived from membranes incubated with β-globin chains showed a spectrin binding defect similar to that seen in genuine α-thalassemic IOVs.[32]

An entirely different abnormality was detected in RBC of β-thalassemic patients. Their protein 4.1 showed a markedly reduced ability (by about 48%) to enhance the binding of normal spectrin to actin and a decreased ability to bind spectrin in a binary interaction as compared to control or α-thalassemic protein 4.1 (FIG. 1).[29] This abnormality might provide an explanation for the different rheological properties described previously. Increased fragility had previously been observed in patients with either protein 4.1 or spectrin defficiency,[33] as well as in patients with defects involving spectrin self association[34] or with abnormalities involving the interaction of spectrin with actin and protein 4.1.[35] Since no quantitative deficiencies were detected in membrane proteins of thalassemic RBC, the increased fragility found in β-, but not α-, thalassemic membranes may have resulted from their functionally defective protein 4.1.

The abnormal function of β-thalassemic protein 4.1 had been demonstrated on isolated molecules of the extracted protein.[29] To study the possibility that β-thalassemic skeletal proteins were altered by the precipitated globin chains *in situ,* the RBC membranes were solubilized with 1 *M* Tris buffer and resolved by gel chromatography.[36] It appeared that α-globin monomers and aggregates (16, 32, and 64 kDa) copurified with the cytoskeletal proteins spectrin, protein 4.1 and actin, which had been shown to function abnormally. Similar studies have yet to be performed in α-thalassemia.

Spectrin, the major cytoskeletal protein, showed mild structural changes in both α- and β-thalassemic RBC. Compared to controls, there was a small increase in the percentage of spectrin oligomers in low ionic strength extracts of thalassemic membranes prepared at 4°C,[29] which could have resulted from the globin present in excess on thalassemic membranes, since hemoglobin is believed to enhance spectrin self-association.[37] In addition, a mild structural change could be seen in spectrin extracts prepared at 37°C, where a fast mobility band (FMB) was detected, migrating approximately in the position of spectrin monomer[38] in a non-denaturing gel system. This FMB suggested that a small amount of thalassemic spectrin had incurred some oxidative damage.[38] Despite these morphological changes in spectrin structure, no alterations from normal could be found in the various functions of spectrin from RBC of either non-Asian α- or non-Asian β-thalassemic patients.[29] Defective spectrin dimer self-association was reported, however, in Asian α- and β-thalassemic patients.[39] In that study, the percent of spectrin present as dimers when extracted from RBC membranes was elevated, and conversion of spectrin dimers to tetramers was reduced. These alterations in the behavior of spectrin might result from

differences not only between α- and β-thalassemia, but also between different mutations within the globin genes, resulting in distinct clinical disease entities in different parts of the world.

OXIDATIVE DAMAGE

Alterations in structure and function of thalassemic membrane proteins may result not only from direct precipitation of the different globin chains, but also from oxidative damage, to which these cells are very prone.[3] In addition to the susceptibility of membrane proteins to oxidative damage, evidence for increased lipid peroxidation has also been documented. Rachmilewitz *et al.* had already shown by 1976 that RBC from β-thalassemic patients contained a reduced percentage of phosphati-

TABLE 1. Differences in Globin Chain Precipitation and RBC Membrane Functions in α- and β-Thalassemia

Property	α-Thalassemia (Hb H)	β-Thalassemia
Deficient globin chain	α chain	β chain
Excess globin chain	β tetramers	α monomers
Inclusion body formation		
Rate	Usually late in RBC lifetime	Early in erythropoiesis
Localization	Membrane associated	Intracytoplasmic and intranuclear
Mechanical properties		
Rigidity	Increased	Increased
Fragility	Normal	Increased
Skeletal protein functions	Defective binding of normal spectrin to IOVs[a]	Defective binding of protein 4.1 to spectrin and actin
Erythroblastic protein synthesis	Active	Depressed
Ineffective erythropoiesis	Mild-moderate	Severe
Hemolysis	Mild	Severe

[a]IOVs, inside-out vesicles.

dylethanolamine (PE), increased phosphatidylcholine (PC), a decrease in the polyunsaturated fatty acids, and a twofold increase in malonyldialdehyde (MDA) compared to normal RBC.[40] In a more recent study, Zipser *et al.*[41] showed that β-thalassemic RBC were much less sensitive than controls to lipid hydrolysis with phopholipase A_2. This observation might reflect altered phospholipid organization, with more PC present in the inner phospholipid layer—or in the outer layer, but unavailable to phospholipase A_2. Also in that study, more PE was detected in the outer layer.[41] In a study by Ben-Yashar *et al.* the presence of fluorescent MDA adducts such as PE-PE, phosphatidylserine-phosphatidylserine (PS-PS), and PE-PS was demonstrated when RBC membranes from β-thalassemics were analyzed by thin layer chromatography.[42] These adducts were characteristic products of MDA cross-linking between aminophospholipids.[43] Their presence, together with the appearance of derivatives of cholesterol oxidation in stored thalassemic RBC,[42] further supported the concept that modification of membrane lipids of thalassemic RBC might contribute to the

pathophysiology of severe hemolysis in this disease. So far similar studies have not been conducted in α-thalassemia; such studies will be necessary in order to establish the role of lipid peroxidation in the latter form of the disease.

SUMMARY

The basic pathology in all forms of thalassemia results from the presence of excess unstable globin chains within the pathological RBC, but the pattern and rate of their precipitation is different (TABLE 1, FIG. 1). Consequently, their effects on the RBC membrane components are not the same and may account for the different rheological properties that have been found.

It is possible that the damage incurred by excess β chains in Hb H disease is primarily due to the direct interaction of the large inclusions with some cytoskeletal proteins such as spectrin, ankyrin, and band 3. In β-thalassemia, where excess unstable α chains have already precipitated in young erythroblasts, the main damage might be caused by an excess of free oxygen radicals, which affect in particular protein 4.1.

A search for additional changes and for potential differences in the membrane and cellular properties between the different thalassemic syndromes is warranted in order to understand better the different clinical expression in the various types of the disease. Moreover, when there is a better elucidation of the mechanisms by which the RBC are destroyed, one may look for possible ways and means to prevent these changes, with a consequent extension of the current short life span of the affected RBC.

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PART II. REGULATION OF HUMAN FETAL AND ADULT HEMOGLOBIN PRODUCTION

Analysis of Human γ-To-β Switching in Transgenic Mice[a]

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INTRODUCTION

During the last forty years a variety of approaches have been used for the analysis of globin gene switching (reviewed in Ref. 1). These include *in vivo* observations in various species, pharmacologic and hormonal manipulations of fetuses, adult-to-fetus and fetus-to-adult hemopoietic cell transplantations, production of chimeric animals, hemopoietic progenitor cell cultures, and production of heterospecific hybrids. Most recently, insights into the molecular control of switching have been obtained with studies in transgenic mice. In the mouse, the embryonic genes of the β locus, ϵ^y and β^{h1}, are expressed in the yolk sac stage of erythropoiesis, while the adult genes β^{minor} and β^{major} are expressed when the definitive stage of hematopoiesis starts in the fetal liver. Experiments with transgenic mice containing fragments of γ- or β-globin genes and their proximal flanking sequences showed that sequences important for developmental-stage specificity of globin gene expression are located in the immediate vicinity of the γ or β genes or within the genes themselves.[2-5] Thus, the human γ gene is expressed only in the primitive, yolk sac cells and not in the definitive fetal liver cells.[2,3] In contrast, the β gene is expressed in the definitive cells of the erythroid liver, but not in the yolk sac cells.[4,5] However, in all these experiments the level of expression was low and dependent on the site of integration into the host genome, suggesting that critical regulatory elements were missing from the transgenic fragments.

Studies of the β-globin locus chromatin have revealed two types of DNase I–hypersensitive sites. Sites of the first type are located at the globin promoters of the expressed globin genes and thus are developmentally specific.[6] Sites of the second type are located 6–22 kb upstream of the ε-globin gene and are developmentally stable.[7,8] Studies in somatic cell hybrids,[9] transgenic mice,[10] and cell lines[11] have

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shown that the region containing these developmentally stable hypersensitive sites is important for high-level position-independent β-globin expression. This region, called the locus activation region (LAR) or dominant control region (DCR) presumably activates the expression of the whole β-globin locus by organizing the locus into an active chromatin domain. The LAR has been shown to confer high-level erythroid-specific expression on heterologous promoters,[12] as well as on lymphoid genes[13] or housekeeping genes.[14] Given this dominant nature of the LAR, appropriate developmental regulation of the fetal and adult globin genes necessitates that they escape the influence of the LAR at the developmental stage at which they are quiescent. The goal of our studies was to explore how the globin locus is developmentally controlled in the presence of the LAR.

RESULTS AND DISCUSSION

The LAR Alters the Developmental Regulation of Fetal Globin Genes

We used an LAR construct containing the four erythroid-specific DNase I–hypersensitive sites from the 5′ region of the β-globin locus within a 2.5-kb fragment.[11] This construct, termed micro-LAR (μLAR),[11] was linked to a 3.3-kb $^{A}\gamma$-globin gene containing a fragment which has been previously shown to be developmentally controlled in transgenic mice.[2,3] The expression of the transgene was analyzed in fetal blood and fetal liver erythroid cells.

The blood of a day 10–11 fetus is derived exclusively from the yolk sac islands and is composed of nucleated embryonic erythroblasts; these embryonic erythroblasts

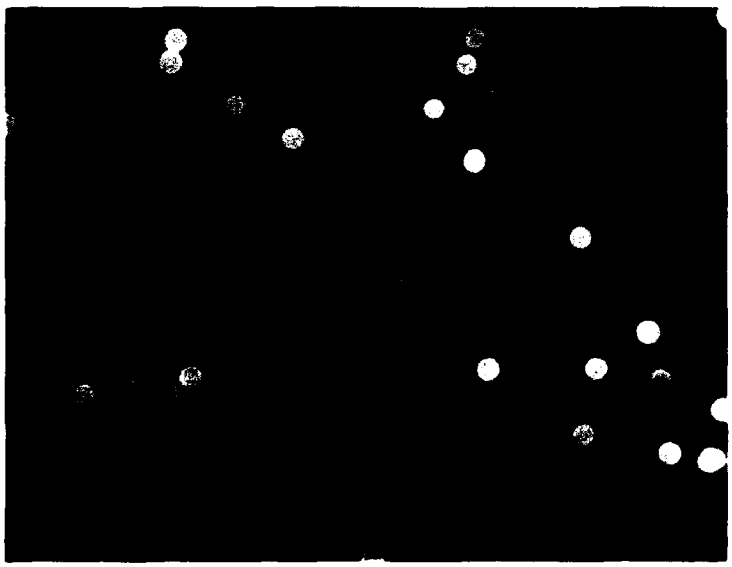

FIGURE 1. Peripheral blood of an adult μLAR-$^{A}\gamma$ transgenic animal. Cytocentrifuge smears were labeled with anti–human γ chain monoclonal antibody. Note that human γ-globin is expressed in the erythrocytes of an adult animal.

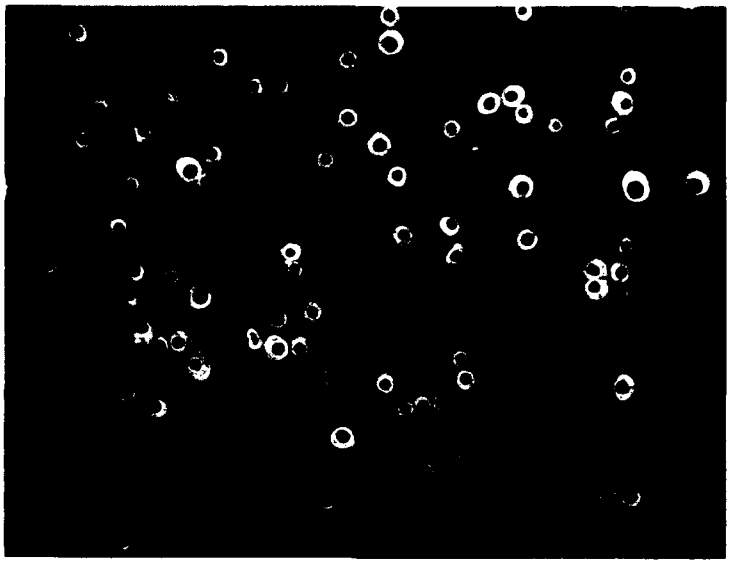

FIGURE 2. Labeling of day-11 placental blood of a μLAR-β transgenic animal with anti-human β chain fluorescent antibodies. Note the expression of human β-globin in the embryonic erythroblasts. Unlabeled smaller cells in this preparation represent contamination by maternal red cells.

express human fetal globin. Starting at day 11, fetal liver–origin non-nucleated red cells appear in the circulation, and the blood of the fetus is then composed of a mixture of definitive, non-nucleated red cells and of primitive, nucleated red cells. Immunofluorescent labeling of blood from a 14-day fetus showed expression of human γ-globin in both the embryonic and the definitive erythroid cells. Human γ-globin continued to be produced in the erythrocytes of the adult mice. In these animals, the level of γ-globin mRNA was about 1/3 to 1/5 of that in the fetus, and 20–30% of the erythrocytes were labeled by the anti–γ chain fluorescent antibodies (FIG. 1). These results suggest that the LAR overrides the developmental control of the γ-globin gene so that there is inappropriate expression of this gene in the adult cells.

The LAR Overrides the Developmental Control of β-Globin Genes

To investigate whether the LAR affects the developmental control of the human β-globin gene, we used a construct in which the 2.5-kb μLAR cassette was linked to a 4.8-kb fragment containing the β-globin gene. Previous studies have shown that this fragment is developmentally regulated in transgenic mice, being expressed only in definitive and not in primitive erythroid cells.[4,5] The μLAR-β transgenic mice expressed human β-globin in the adult red cells. In addition, as shown in FIGURE 2, the μLAR transgenic animals express human β-globin in the primitive, yolk sac–origin erythroid cells, indicating that the LAR overrides the developmental control of the β-globin gene.

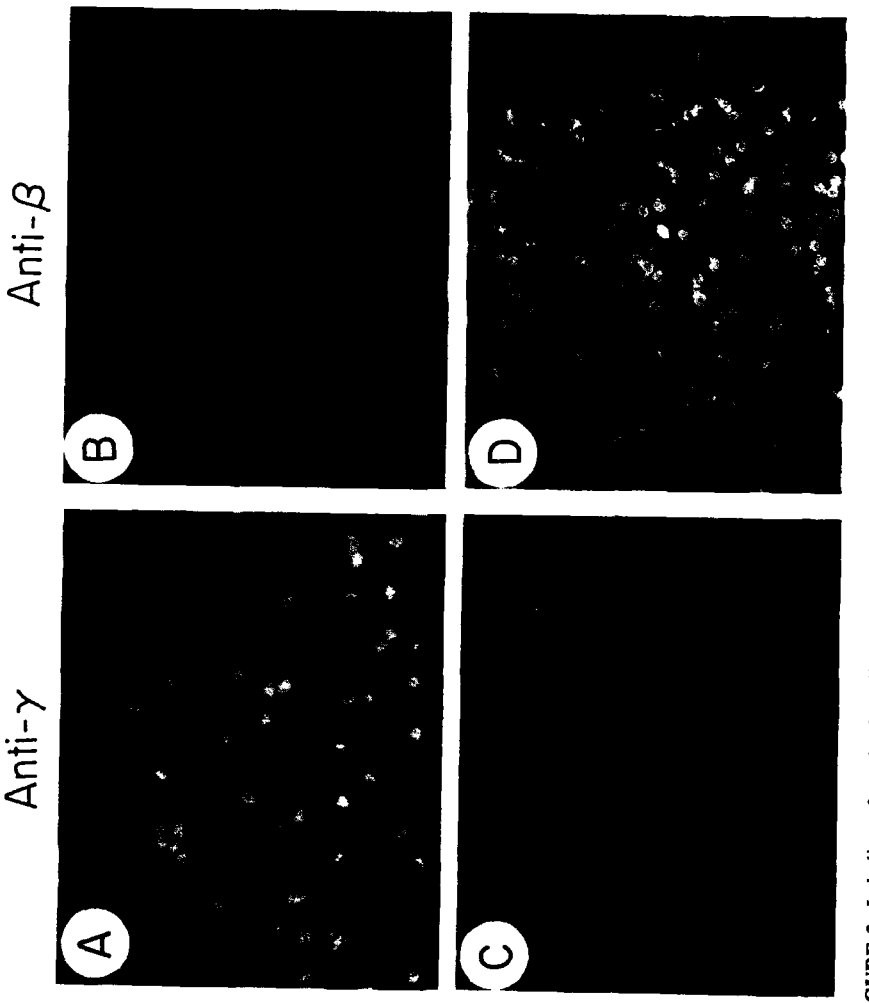

FIGURE 3. Labeling of erythroid cells from μLAR-^γψβδβ transgenic animals using anti-human β or anti-human γ chain fluorescent antibodies. (**A and B**) Embryonic blood, (**C and D**) adult blood.

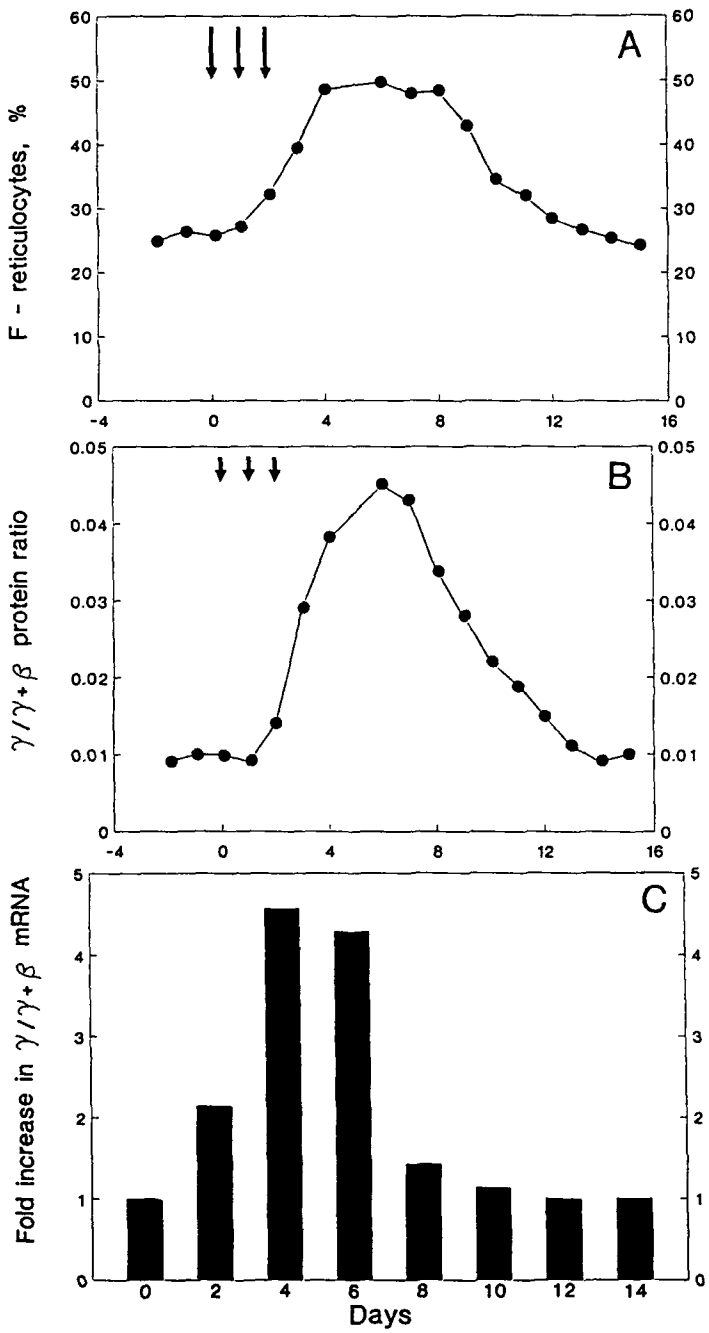

FIGURE 4. Treatment of a μLAR-^γ transgenic mouse with erythropoietin (*arrows:* 3000 IU/kg, twice daily). Notice the induction of γ-globin expression, as indicated in the rise of (**A**) F reticulocytes and (**B, C**) γ/(γ + β) ratios.

Restoration of Developmental Control in the μLAR-$^{A}\gamma\psi\beta\delta\beta$ Construct

The findings with the μLAR-$^{A}\gamma$ and μLAR-β constructs raise a paradox, since both β and γ genes are developmentally regulated in their normal chromosomal context, which contains the LAR. To mimic the genomic organization of the β locus, 29 kb of contiguous β-locus sequence from $^{A}\gamma$ up to and including the β-globin gene was linked to the μLAR. Transgenic mice containing the μLAR-$^{A}\gamma\psi\beta\delta\beta$ contructs expressed γ- but not β-globin genes in the embryonic red cells (FIGS. 3A and B). Both β and γ genes were expressed in the cells of the fetal liver. There was abundant β-globin expression and low γ-globin expression in the red cells of the definitive erythropoiesis (FIGS. 3C and D).

These results showed that correct globin gene developmental regulation is achieved when both β and γ genes together with LAR are present in a construct. Presumably, sequences present in the $^{A}\gamma\psi\beta\delta\beta$ fragment prevent the interaction between LAR and the β gene in the embryonic cells, and LAR and the γ gene in the definitive cells. Alternatively, at each developmental stage, *trans*-acting factors may interact with the globin genes, changing their relative ability to interact with the LAR.[15] A similar competitive model has been previously proposed for explaining hemoglobin switching in the chicken.[16,17]

Induction of γ Expression in Adult Transgenic Mice

Several compounds and physiological conditions have been found to stimulate fetal hemoglobin production in the adult.[1] These compounds have been detected with studies of patients or with experiments in primates. We tested whether the μLAR-$^{A}\gamma$ mice could be used as an animal model for studying induction of Hb F in the adult.

Heterozygous μLAR-$^{A}\gamma$ transgenic animals were used. Administration of doses of erythropoietin known to induce Hb F in baboons[18] resulted in a sharp increase of reticulocytes, F reticulocytes, and $\gamma/(\gamma + \beta)$ mRNA and protein ratios (FIG. 4). Induction of γ-globin expression was also obtained when these animals were treated with 5-azacytidine, hydroxyurea, or sodium butyrate.[19] These results suggest that transgenic mice provide a model system for the investigation of the induction of Hb F in the adult.

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Stopping the Biologic Clock for Globin Gene Switching[a]

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The developmental switch from production of γ-globin to β-globin results in significant morbidity when the β-globin genes are defective. The globin switch has therefore been extensively studied. It appears to be set on a biologic clock and proceeds solely on the basis of gestational age, despite the site of blood production.[1] We previously found that this developmental gene switch is delayed in human fetuses developing in the presence of maternal diabetes.[2] A number of metabolites present in abnormal concentrations in these infants were therefore tested for effects on globin expression. One metabolite, α-amino-*n*-butyric acid (ABA), was found to enhance γ-globin and inhibit β-globin expression in cultured neonatal erythroid cells.[3] Further investigation showed that ABA, sodium butyrate, and similar compounds can enhance γ-globin expression in cultured cells of patients with β-globin diseases and that these agents can delay and inhibit the globin switch in an *in vivo* fetal animal model. To explore possible mechanisms of action of these compounds, the effects of butyric acid on histone acetylation in erythroid cells and the effects of butyrate analogues on the γ-globin promoter linked to reporter genes were examined.

MATERIALS AND METHODS

Erythroid Progenitor Cultures

Peripheral blood was collected into preservative-free heparin. The mononuclear cells were separated on Ficoll-Hypaque, washed, and cultured in Iscove's modified

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Dulbecco's medium (IMDM) with methylcellulose and with 2.5 U/ml erythropoietin (Terry Fox Laboratories, Vancouver, Canada) and 5 U/ml of granulocyte-macrophage colony stimulating factor (GM-CSF: Amgen, Thousand Oaks, CA), with and without sodium butyrate or 10 other compounds consisting of slight modifications of butyric acid, at concentrations of 0.1–0.5 mM, as previously described.[4] On day 12–13 of cell culture, BFU-e (burst-forming unit–erythroid) were harvested and labeled with 25 μCi of lyophilized [^{3}H]leucine in 250 μl of leucine-free minimum essential medium with 15% fetal calf serum and the same concentration of analogues in which the cells were cultured. Lysates of cultured erythroblasts were electrophoresed on acid-Triton-urea gels, and autoradiographs were prepared and scanned on a laser densitometer to quantitate proportions of α- and non–α-globin chains as previously described.[4]

Analysis of Histone Acetylation Patterns

Histone acetylation patterns in the presence and absence of butyrate analogues were examined, as the well-described induction of histone hyperacetylation by butyrate is one potential mechanism which might explain an effect on maintenance of active chromatin structure in the region of the γ-globin genes during gestation.[5-9] Fetal liver and K562 cells were utilized in order to study a population of greater than 90% erythroid cells. Single-cell suspensions of 10^7 cells each were incubated at 37°C in 5% CO_2 in Iscove's medium with 10% fetal calf serum alone or with 20 m*M* sodium butyrate, halogenated compounds designated 901409 and 907388, L-α-amino-*n*-butyric acid, or D-chloro-alanine (designated D-594). After 48 h of treatment, nuclei and acid extracts were prepared and the samples were dialyzed into 1 *M* acetic acid. Protease inhibitors and sodium butyrate were added to the preparations at all steps after incubation. 44 μg of the final extract was electrophoresed on acid-urea gels to resolve acetylated histones as previously described.[10]

Fetal Ovine Studies

Since the globin gene switch occurs before birth in animal models, we tested butyrate analogues in an ovine fetal model. We chose this model because the ovine fetus tolerates vascular catheterization for blood sampling and infusions with the lowest experimental morbidity rate. Venous and arterial catheters were placed in ovine fetuses by hysterotomy as previously described.[11] Butyric acid, neutralized to pH 7.4, and two analogues of butyric acid with minor molecular substitutions were infused into the fetuses using a peristaltic pump suspended on the mother's side. Blood was sampled regularly, and assay of globin chain synthesis was performed on [^{3}H]leucine-labeled reticulocytes.[11] Results were compared with globin synthesis in ovine fetuses who were similarly operated on *in utero,* including some which were infused with normal saline at the same rate.

Globin Promoter–Reporter Gene Transient and Stable Expression Assays

Chimeric genes consisting of the γ- and β-globin promoter regions linked to the reporter genes *neo* and human growth hormone (*HGH*) were tested in stable and transient expression assays, respectively, with and without butyrate analogues. Survival in the presence of G418 was compared in the presence and absence of

butyrate treatment of cells. Chimeric genes consisting of the γ-globin promoter region linked to the *HGH* reporter gene were transfected into K562 cells by electroporation, and transient expression of HGH was assayed at 72 h and compared in the presence and absence of butyrate treatment of the transfected cells.[12] One construction (*γ), generously provided by S. Orkin, includes a DNase-hypersensitive site which has been shown to be critical for β-globin expression.[13-16] β- and γ-globin promoter-*neo* constructs were transfected into Rauscher murine erythroleukemia cells, an erythropoietin-responsive cell line which produces high levels of adult murine globin.[17]

RESULTS

Cells from 25 patients with sickle syndromes or β-thalassemia were cultured with and without butyric acid compounds. In two-thirds of patient samples, butyrate analogues increased γ-globin expression by a mean of 12% above the γ-globin production found in control cultures from the same subject. A typical densitometry scan of an autoradiogram from BFU-e cultured from a patient with transfusion-dependent homozygous β-thalassemia is shown in FIGURE 1. There was some γ-globin expression in untreated erythroid cells. In these cultured cells, as in the patient, β-globin was not detectable. Addition of sodium butyrate increased γ-globin expression and improved the α:non–α-globin ratio by 36%, and the effect was seen on both $^{A}\gamma$- and $^{G}\gamma$-globin genes. Compounds which were slight modifications of butyric acid were also effective in increasing γ-globin synthesis.

In an attempt to confirm that butyric acid delays the globin gene switch, an *in vivo* fetal ovine model was utilized. Infusions of sodium butyrate into catheterized ovine fetuses delayed the γ → β globin switch in three of four fetuses. This effect was most

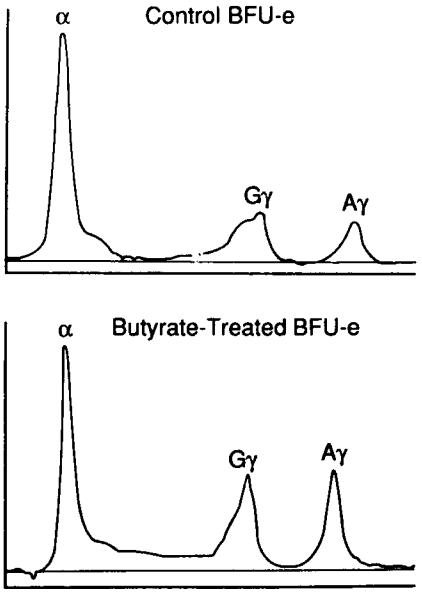

FIGURE 1. Densitometric scans of globin produced by erythroid cultures from a patient with homozygous β-thalassemia. Untreated cells produce some $^{G}\gamma$- and $^{A}\gamma$-globin and no β-globin (**top panel**). Addition of sodium butyrate increased expression of both γ-globin genes and improved the α:non–α-globin ratio by 36% (**bottom panel**). (From Perrine *et al.*[4] Reprinted from *Blood* by permission of Grune and Stratton, Inc.)

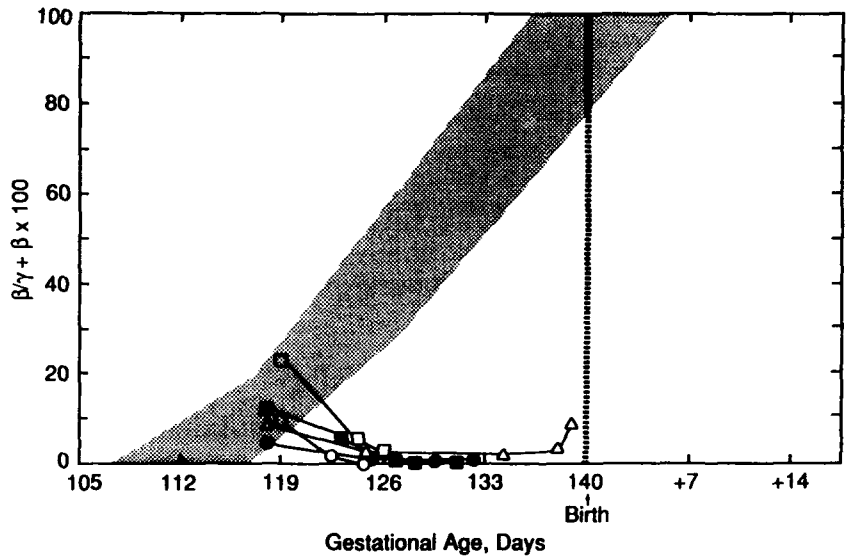

FIGURE 2. β-globin synthesis in normal and compound 907388–treated fetal lambs. The rise in β-globin synthesis in normal fetuses at the end of gestation is shown in the *shaded area;* β-globin synthesis in fetuses treated with the butyrate analogue 907338 *in utero* is shown by the *connected lines.* Adult globin expression was profoundly inhibited by infusions of this compound.

prolonged when the infusions were begun before the β-globin level was greater than 10–15% of the non–α-globin level. In one fetus, β-globin was low at the start of treatment; at term, after three weeks of butyrate therapy, there was no adult (β) globin detectable, and this fetus produced 100% γ-globin. When the infusions were begun in a lamb with greater than 40% β-globin production, the globin switch was not delayed.

In an attempt to increase efficacy by prolonging the half-life of butyrate, two compounds with halogen-ion substitutions, each designed to decrease metabolism of the compound as an energy source, were infused into fetal lambs. One compound produced mild acidosis. In fetuses infused with a second analogue, designated 907388, profound inhibition and even reversal of adult globin production was found in all fetuses treated. These results are illustrated in FIGURE 2, which also shows the switching process in controls.

Because butyrate has been shown to affect histone acetylation patterns in other systems by inhibiting histone deacetylase, we examined whether such an activity might be involved in its action on γ-globin expression. We therefore compared the activity of various modified butyrate compounds both for their activity in stimulating γ-globin expression and for any increase in histone acetylation. The degree of acetylation induced by the compounds correlated with the degree of stimulation of γ-globin synthesis for four of five analogues tested (data not shown).

Evaluation of a possible effect of butyrate on the γ-globin promoter was examined in stable and transient expression assays, using the neomycin resistance gene as reporter in the stable expression assays and the human growth hormone gene as a reporter in the transient expression assays. Results from the transient expression assay are summarized in TABLE 1. The construct *γ includes a DNase-hypersensitive

site which has been shown by Tuan, Grosveld, Curtin, and co-workers[14-16] to be a critical region of the dominant control region for β-globin expression. Addition of this site produced stronger promoter activity, which did not respond to butyrate. The addition of butyrate to cells transfected with β-globin constructs had no effect on expression in these cells, which are permissive to expression of either adult or fetal globin. However, in both the transient and stable expression systems, addition of butyrate to cells transfected with the γ-globin promoter–reporter constructs increased the expression of the γ-globin promoter–driven reporter genes by threefold. This was similar to the increase in expression induced by replacing the normal γ-globin promoter with an HPFH (hereditary persistence of fetal hemoglobin) promoter, as reported by Orkin and co-workers.[13]

TABLE 1. Effect of Butyrate on the Expression of γ-Globin Promoter–Growth Hormone Chimeric Genes Transfected into K562 Cells

	Expression (ng/ml)	
Chimeric Gene[a]	Control	+ Butyrate
β–Pro-GH	0.5	0.3
γ–Pro-GH	5.0	20.0
*γ–Pro-GH	40.0	35.0

[a]Constructs are designed to assess expression of the human growth hormone gene as driven by the β- or γ-globin promoter. The construct *γ includes DNase-hypersensitive site II, which produced stronger promoter activity and did not respond to butyrate.

DISCUSSION

These data demonstrate that a metabolite found in elevated concentrations in the plasma of infants of diabetic mothers, α-amino-*n*-butyric acid, and similar compounds, can increase γ-globin and inhibit β-globin expression in erythroid cells in both *in vitro* and *in vivo* experimental systems. It is therefore likely that this metabolite causes the delayed globin gene switch in these infants, interrupting a biologic clock. Furthermore, when added to the erythroid cells of patients with abnormal β-globin genes, an increase in γ-globin production occurs even in short-term cultures. The amount of enhancement found, up to 40% over control levels, is a degree that could be useful therapeutically if it were achieved in human cells *in vivo*.

It is interesting that the same compounds can inhibit and even reverse the fetal-to-adult globin switch in fetal sheep, as the sheep fetal globin gene is structurally somewhat different from the human fetal globin gene. However, butyrate was shown by Ginder and colleagues[5] to reactivate an embryonic globin gene in adult chickens when given with 5-azacytidine. The action of these compounds may therefore be on several processes related to the chromatin structure required for globin gene regulation, rather than on enhancement of the expression of a single globin gene. Because butyrate is known to inhibit histone deacetylase and to increase the acetylation of histones in other systems,[5-9] we considered that this activity could be involved in the action of butyrate on γ-globin expression. We therefore compared the effects of these compounds on histone acetylation with their effects on γ-globin expression. Finding a strong correlation between the two activities suggests that an effect on histone acetylation may indeed contribute to maintaining γ-globin expression during that part of the gestational time frame in which it is normally suppressed.

In summary, these data indicate that the normally fixed $\gamma \rightarrow \beta$-globin gene switch can be inhibited and even reversed by butyric acid and similar compounds. These agents can enhance γ-globin expression in cultured human erythroid cells to a degree that could greatly ameliorate the α:non-α-globin imbalance that occurs in the β-thalassemias. Thus, although formal toxicity testing must still be done and frequent administration of these compounds would be required, this class of compounds appears to provide a potential pharmacologic means of decreasing the expression of abnormal β-globin genes and replacing their function with fetal globin in patients afflicted with the β-thalassemias and hemoglobinopathies.

SUMMARY

The developmental switch from production of fetal (γ) to adult (β) globin occurs on a normally set biologic clock which proceeds even if expression of the adult (β) globin genes is defective and produces little or no protein, as in the β-thalassemias. Preventing or reversing the globin gene switch could provide a way of keeping the abnormal globin genes "silent" and maintaining expression of the fetal globin gene. We have identified a class of agents which, when present in elevated plasma concentrations during gestation, inhibits the $\gamma \rightarrow \beta$-globin gene switch in developing humans. Further investigation has shown that butyric acid and related compounds can increase γ-globin and decrease β-globin expression in cultured erythroid cells of patients with β-thalassemia. Butyrate compounds were therefore infused in an *in vivo* fetal animal model, and the globin switch was inhibited and even reversed in some fetal lambs. Histone hyperacetylation, which maintains active chromatin structure, and an effect on the γ-globin promoter appear to be mechanisms of action involved. These data suggest that inhibiting expression of abnormal β-globin genes by pharmacologic means may in the future be possible for treatment of individuals with β-globin disorders.

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Localization and Characterization of the DNase I–Hypersensitive Site II (HS II) Enhancer

A Critical Regulatory Element within the β-Globin Locus–Activating Region

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INTRODUCTION

An important advance in the understanding of globin gene regulation has been the description of distant regulatory elements flanking the β-globin gene cluster. These elements were first noted to be associated with DNase I hypersensitivity in isolated erythroid nuclei.[1-2] Four major hypersensitive sites occur 18.0 kb (HS IV), 14.7 kb (HS III), 10.9 kb (HS II), and 6.1 kb (HS I) upstream of the ε-globin cap site and one site occurs 21.3 kb (HS VI) downstream of the β-globin gene. The first evidence suggesting the importance of these regions was the description of the Dutch γδβ-thalassemia mutation, in which deletion of the upstream region caused inactivation of an intact β-globin gene.[3-4] Experimental evidence has been obtained confirming the critical role of these sequences in globin gene regulation. Expression of transfected globin genes in transgenic mice and tissue culture cells is profoundly augmented by these sequences.[5-11] This region has been designated the locus-activating region (LAR) or the dominant control region (DCR), reflecting its influence on expression of the genes within the β-globin gene cluster.

Definition of the active elements within the LAR should provide some understanding of the mechanism of transcriptional activation by this region and allow inclusion of these elements within retroviral vectors used for gene therapy. This formidable task involves defining the minimally active elements within the 30 kb of chromatin encompassing the hypersensitive sites. Some progress toward this goal has already been achieved. Fragments from this region ranging in size from 882 bp to 13 kb have been shown to considerably increase the transcriptional activity of transfected globin genes.[6,9-12]

We chose to study these upstream sites in a construct linked to a marked γ-globin gene in stably transfected K562 cells. Based on these experiments and work from Tuan *et al.* which demonstrates a powerful enhancer within HS II,[13] our studies examined HS II in detail using a transient expression assay system. These assays localized the HS II enhancer to a 20-bp sequence containing tandem repeats for the

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activating protein 1 (AP-1) consensus sequence.[14-15] This minimal enhancer is shown to be necessary and sufficient for the increased expression of γ-globin seen with hemin-induced erythroid maturation of K562 cells. DNaseI protection and gel mobility shift assays demonstrate formation of a multimeric protein complex on this enhancer element that is necessary for high level activity. These results localize and characterize an important discrete element within the β-globin LAR.

METHODS

Stable Expression Assay

K562 cells were electroporated with linearized plasmids containing LAR elements cloned upstream of a $^{A}\gamma$-globin gene marked with a 6-bp deletion in the first exon. This gene included 410 bp of 5′ flanking sequence and 3′ flanking sequence extending 190 bp downstream of the polyadenylation signal. A 1.1-kb neomycin resistance transcription unit was linked downstream of the globin gene in a pUC-based vector. Clones were selected in G418 and pooled so that for each construct 150 clones in 4–10 populations were analyzed. The polymerase chain reaction (PCR) assay used to measure RNA ratios is described elsewhere.[16] Southern blot analysis was done to compare the copy number of the transfected gene to that of the endogenous γ-globin genes. RNA and DNA ratios were quantified by scanning autoradiograms in the linear exposure range with a laser densitometer.

Transient Expression Assay

K562 cells were electroporated with plasmids containing LAR elements cloned upstream of a chimeric reporter gene consisting of a 260-bp γ-globin promoter linked to luciferase coding sequences. A cotransfected B19 promoter–chloramphenical acetyltransferase (CAT) gene was used as an internal control for transfection efficiency. Deletion mutagenesis of the 1.45-kb HS II fragment is described elsewhere.[17] Primer extension analysis showed that the transcript from the transfected gene was correctly initiated and that RNA levels corresponded to luciferase activity.

Gel Mobility Shift Assays

Nuclear extracts were prepared by the method of Dignam *et al.*[18] in the presence of phenylmethylsulfonyl fluoride, aprotinin, pepstatin, and leupeptin. Probes were prepared from oligonucleotides subcloned into a pUC vector, labeled with Klenow fragment, and purified over 12% polyacrylamide gels. Gel shift assays were performed with 20,000 cpm of probe added last to a 20-μl reaction mixture containing varying amounts of nuclear extracts and 1 μg of poly(dI-dC). The final ionic composition of the reaction mixture was 20 m*M* HEPES, 60 m*M* KCl, 0.2 m*M* EDTA, 6 m*M* $MgCl_2$, 0.5 m*M* dithiothreitol, 10% glycerol, and 6% polyethelylene glycol. Double-stranded synthetic oligonucleotides were used as cold competitors in some reactions and were added with the probe. Samples were incubated at 25°C for 30 min and then run on a 4% non-denaturing polyacrylamide gel.

RESULTS

HS II Is Necessary for High-Level Expression of a Stably Transfected γ-Globin Gene in K562 Cells

Fragments encompassing each of the three upstream hypersensitive sites were assayed alone or in combination in stably transfected K562 cell populations by comparing expression of a marked γ-globin gene to which they were linked with that of the endogenous γ-globin genes. RNA was analyzed by PCR amplification of cDNA derived from pooled clones selected in G418. This assay was shown to correlate with standard RNase protection assays over a wide range of levels of

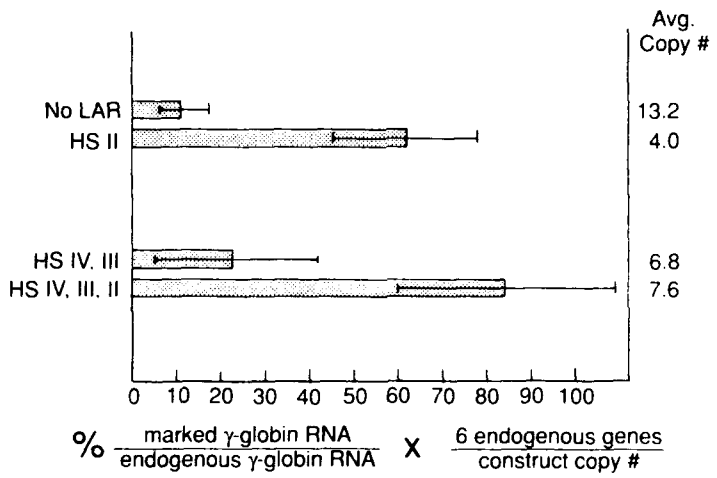

FIGURE 1. The effect of LAR sequences on γ-globin gene expression in uninduced stably transfected K562 cells. Each *shaded bar* represents the average copy number–corrected expression of the transfected γ-globin gene relative to one endogenous γ-globin gene. The LAR fragments linked to the transfected gene are indicated on the *left*. For each construct, 150 clones were analyzed in pools as described in the text. *Error bars* represent one standard deviation from the mean. The average transfected gene copy number for each experiment is shown on the *right*. LAR fragments assayed were as follows: HS IV, 1.4-kb *Bam*H I–*Sph* I fragment; HS III, 1.9-kb *Hin*d III fragment; HS II, 1.45-kb *Kpn* I–*Bgl* II fragment.

marked gene expression. The activity of LAR fragments was defined as the ratio (calculated as a percentage of endogenous activity) of marked gene expression to endogenous gene expression after correction for transfected gene copy number. FIGURE 1 shows that a 1.45-kb fragment containing HS II is required for high-level expression of the marked gene. With the addition of HS II, RNA levels from the linked γ-globin gene increased from 11% of that of a single endogenous gene to 61%. Although HS IV and HS III together were relatively inactive (23%), addition of HS II to this construct increased expression to levels approximating that of the endogenous genes (84%).

HS II Contains a Powerful Enhancer That Localizes to a 20-bp Sequence Containing Tandem AP-1 Binding Sites

Transient expression of a reporter plasmid containing the γ-globin promoter linked to luciferase coding sequences was assayed in uninduced K562 cells. The

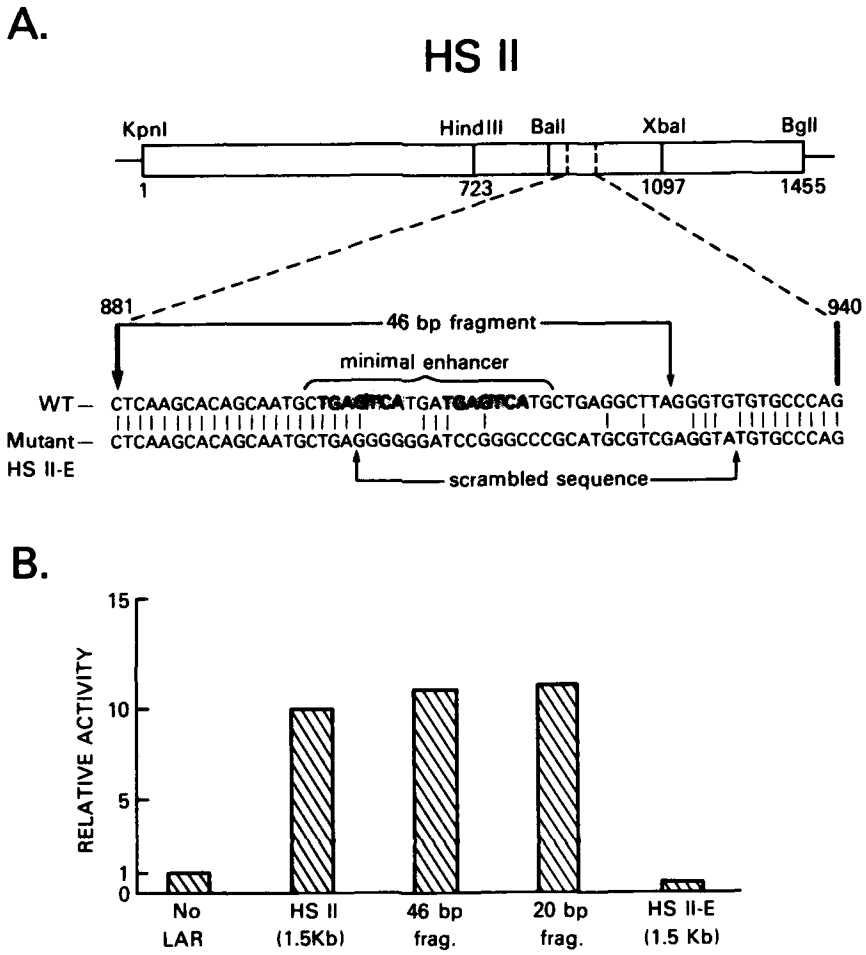

FIGURE 2. (**Panel A**) Location and sequence of the HS II minimal enhancer. The *Kpn* I–*Bgl* II fragment containing HS II is numbered 1–1455 for simplicity, but these numbers correspond to Gene Bank coordinates 7764 and 9218, respectively. The wild-type sequence from 881 to 940 is shown (WT) to indicate the position and sequence of the minimal enhancer. The 46-bp fragment containing the minimal enhancer and the mutant HS II fragment containing the disrupted enhancer (HS II-E) are also shown. The two *shaded boxes* show the tandem AP-1 consensus sequences. (**Panel B**) Relative transient expression of the luciferase reporter gene linked to the indicated LAR fragments in uninduced K562 cells. The reporter gene with no LAR was assigned a relative activity of one. Each *bar* represents the average of two or three experiments.

1.45-kb HS II fragment increased activity of the reporter gene about 10-fold over baseline (FIG. 2B). Exonuclease III digestion provided a series of 3′ and 5′ deletion mutants which mapped the enhancer to a 76-bp fragment. Cloned synthetic oligonucleotides were used to further localize this element to a 20-bp sequence (FIG. 2A). Inspection of this sequence revealed a tandem repeat for the AP-1 consensus sequence. To prove that no other sequences within the 1.45-kb HS II fragment had enhancing activity, we constructed a mutant (HS II-E) that replaced sequences within the minimal enhancer with plasmid polylinker ablating both AP-1 sites but leaving the total size of the fragment unchanged (FIG. 2A). This mutant was inactive, unequivocally demonstrating that the HS II enhancer is a discrete element (FIG. 2B).

The HS II Enhancer Is Necessary and Sufficient to Confer Inducible Expression on a Linked γ-Globin Gene in Stably Transfected K562 Cells

One important aspect of transcriptional regulation of globin genes is the large increase in globin expression seen as an early erythroid cell matures and differentiates. Hemin is a chemical inducer of erythroid maturation in K562 and MEL cells. When K562 cells are cultured in hemin, they undergo a limited and reversible differentiation program with increased synthesis of globin mRNA and other erythroid-specific gene products.[19-22] We made the unexpected observation that induction of γ-globin gene transcription requires the HS II minimal enhancer. Inducibility of a stably transfected marked gene was compared to endogenous globin gene inducibility by comparing the marked-to-endogenous γ-globin RNA ratios from uninduced versus induced pools of clones. Each pool was split, and one-half of each population was cultured in the presence of 20 μM hemin for 3 days. FIGURE 3A shows that the transfected marked gene without linked LAR elements was not induced with hemin, in contrast to the large increase in expression of the endogenous globin genes. When the marked gene was linked to the 1.45-kb HS II fragment, the transfected gene was induced at least as well as the endogenous genes, as shown by a slight increase in the marked-to-endogenous γ-globin RNA ratios with induction (FIG. 3B). When the mutant HS II-E was tested in combination with sites IV and III, the averaged marked-to-endogenous γ-globin RNA ratios fell with induction from 20% to 7% ($p = 0.001$), showing that the HS II enhancer is required for induced expression (FIG. 3C). Adding the 46-bp fragment containing the minimal enhancer to sites IV and III reconstituted high-level inducible expression (FIG. 3D). The 46-bp fragment alone conferred inducible expression to the marked gene, although the absolute level of expression was lower than when sites IV and III were included in the construct (FIG. 3E). Inducible expression was also noted in the transient assay system by an increase in reporter gene activity 170-fold over uninduced activity without the enhancer (see FIG. 5B, below).

Protein Complex Formation Involving Both AP-1 Sites of the HS II Enhancer Is Necessary for Full Activity

DNase I protection studies showed a strong footprint directly over the minimal enhancer; this was present with both erythroid and non-erythroid nuclear extracts. DNA-protein interactions at the enhancer were further studied by gel retardation assays using the 46-bp enhancer fragment as a probe. Nuclear extracts from uninduced K562 cells gave several retarded bands (FIG. 4A, lane 1), whereas nuclear extracts from induced K562 cells gave a broad, highly retarded band (lane 7).

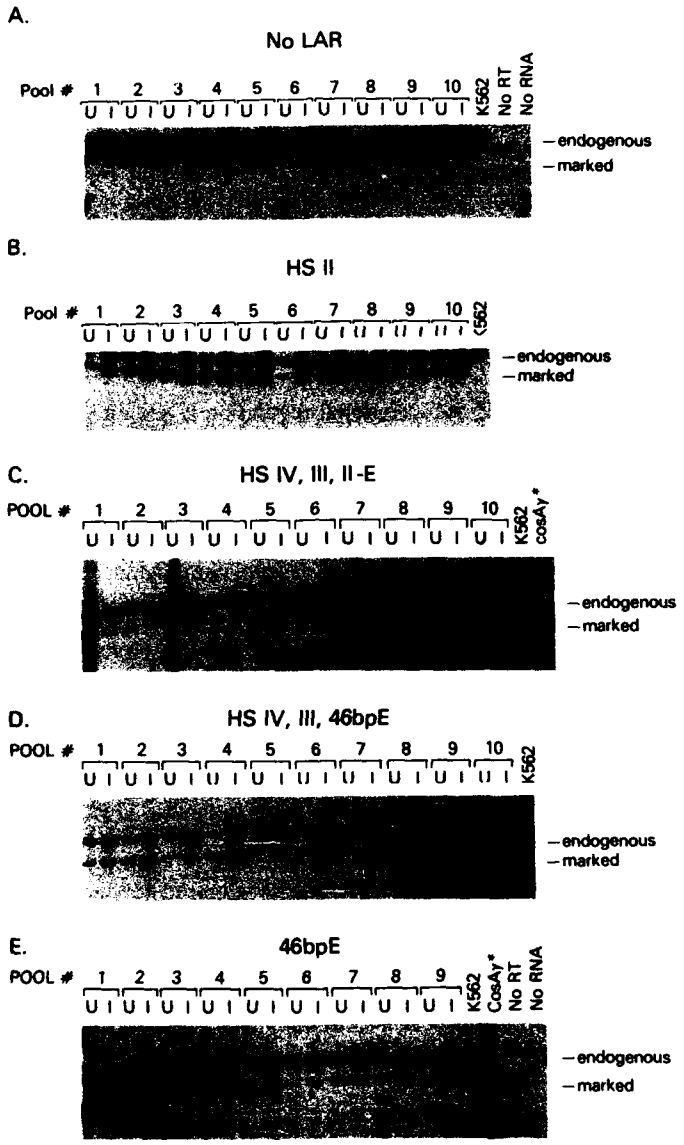

FIGURE 3. RNA analysis by PCR of uninduced and hemin-induced K562 cell pools of clones. Each pool of clones was split, and half of the cells were stimulated with hemin for 3 days as described in the text. (**Panels A, B, C, D, and E**) Each *lane* represents amplified cDNA from 15 to 35 pooled clones for pools containing the indicated LAR fragments linked to the marked globin gene. U, the uninduced aliquot for each pool; I, the hemin-induced aliquot. Controls in the *right-most lanes* are assays of non-transfected K562 cells (K562), Cos cells (which express no endogenous globin) transfected with the marked γ-globin gene (CosAγ*), RNA amplified without a preceding reverse transcriptase reaction (No RT), and samples with no RNA. Positions of endogenous and marked signals are indicated on the *right*.

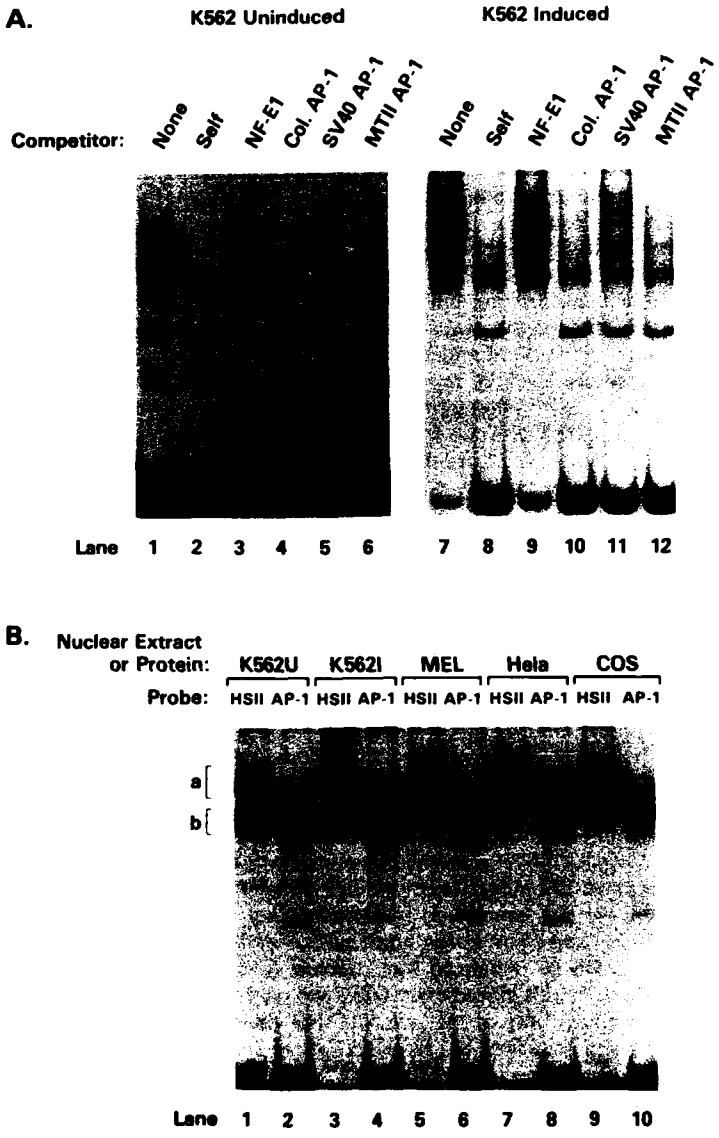

FIGURE 4. Gel mobility shift assays of the HS II enhancer. (**Panel A**) The 46-bp HS II enhancer utilized as probe with 4.4 μg of nuclear extract from uninduced or induced K562. Synthetic oligonucleotides were used as unlabeled competitors in a 200-fold excess. The NF-E1 competitor consisted of the −199 to −163 region of the γ-globin gene promoter that contains a T→C substitution at position −175. The AP-1 competitors were derived from the collagenase gene (Col.), SV40, and metallothionein gene (MTII) enhancers. (**Panel B**) The HS II enhancer–containing probe (HS II) and a single AP-1 site–containing probe (AP-1) compared in alternate lanes. The latter was derived from region II of the chicken β-globin enhancer. These probes were studied against a panel of nuclear extracts (3–5 μg per reaction). The less-retarded bands (b) comigrate with the single AP-1 site probe, suggesting that they represent binding to a single AP-1 site. The highly retarded band (a) represents binding to both AP-1 sites in the HS II enhancer.

Authentic AP-1 binding sites specifically competed in both assays (lanes 4–6 and 10–12). We hypothesized that the highly retarded band seen with K562 nuclear extracts from induced cells represented protein-complex formation involving both AP-1 sites and that this complex formation was necessary for full enhancer activity. Consistent with this, the highly retarded band seen with extracts from induced K562 could be reproduced by increasing the concentration of extract from uninduced cells. Also, the less retarded bands seen with erythroid and non-erythroid extracts comigrated with a probe containing a single AP-1 site from the chicken β-globin enhancer[23] (FIG. 4B, band b).

To further test this hypothesis, we constructed a series of linker scanning mutants disrupting either the 5′ or 3′ AP-1 site (FIG. 5A). When these mutants were tested for enhancer activity in the transient expression assay, a marked reduction in activity was seen when either of the two sites were ablated (FIG. 5B). The activities of mutants 1–6 were markedly diminished in both induced and uninduced cells. Not only were the absolute levels of reporter gene activity reduced by these mutations, but the increase in expression seen with hemin induction was also reduced to 30–70% of that observed with the wild-type enhancer. In contrast, mutant 7, which leaves both AP-1 sites intact, had activity comparable to the wild-type fragment. Gel shift assays using mutant probes 1–6 showed that the highly retarded band characteristic of extract from induced cells was greatly diminished when either AP-1 site was ablated (FIGS. 5C and 5D). In contrast, the mutant 7 probe, which retains both AP-1 sites, showed a gel shift pattern identical to that of the wild-type probe with either extract from induced K562 or from HeLa cells. These data show that complex formation and full enhancer activity requires both AP-1 sites.

DISCUSSION

We have shown that a powerful and discrete enhancer within HS II of the β-globin LAR is required for increased γ-globin transcription with induced maturation of K562 cells. These data suggest a physiologic role for this enhancer in the increased globin gene expression that is characteristic of erythroid cell maturation. It is remarkable that this 20-bp sequence located more than 30 kb from the γ-globin genes is an important globin regulatory element. Recent data from transgenic mouse experiments support this conclusion by showing that this element is necessary for high-level β-globin expression in the adult mouse erythron. When a 67-bp segment that includes the HS II enhancer was deleted from a 732-bp HS II fragment, substantial diminution of β-globin expression was noted.[24] An attractive model is that high-level expression of any of the genes within the β-globin cluster may require interaction of a given gene with the HS II enhancer and that competition among the individual genes for this distant element may be important in hemoglobin switching. The recent observation that transgenic mouse lines containing LAR constructs exhibit a fetal-to-adult switch in expression of the human globin transgenes makes this model testable.[25,26]

Important transcription factors bind to both AP-1 consensus sequences within this enhancer, and formation of this protein complex correlates with activity in expression assays. The identity of the proteins within erythroid cells that interact with the enhancer is still uncertain. The presence of tandem AP-1 binding sites on the enhancer and competition of the gel shift complex with known AP-1 binding sites suggest that members from the Jun and Fos families are able to bind the enhancer. Jun and Fos are families of proteins that bind as heterodimers to the AP-1 consensus sequence.[27,28] These proteins are expressed in many cell types and have been

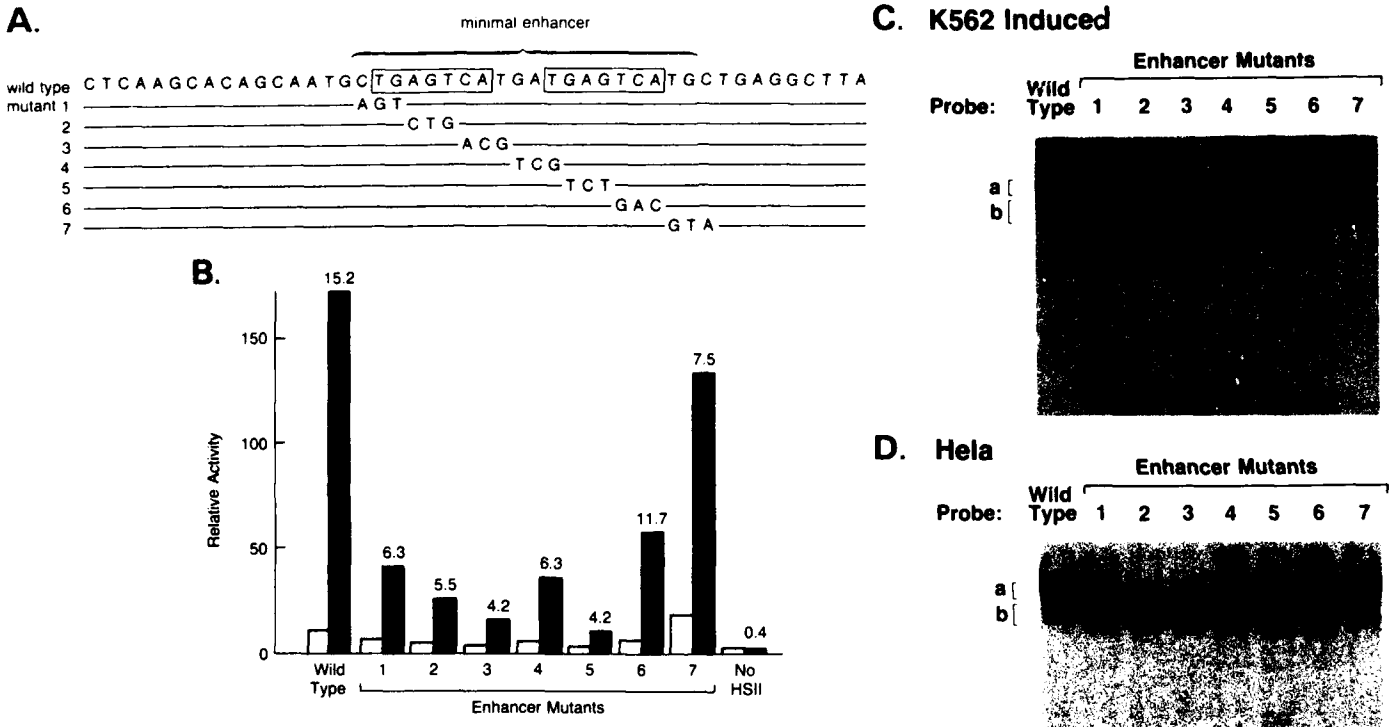

FIGURE 5. Scanning mutants of the HS II enhancer. (**Panel A**) Sequence of the wild-type and mutant enhancer fragments in relation to the two AP-1 binding sites. The tandem AP-1 sites are shown in *boxes*. Seven mutants scanning the enhancer in 3-bp intervals are shown underneath the 46-bp wild-type sequence. Note that only mutant 7 falls completely outside both AP-1 binding sites. (**Panel B**) Activity of the wild-type and mutant enhancers relative to an enhancerless reporter plasmid (no HS II) in uninduced (*open bars*) and induced (*solid bars*) K562 cells. The fold increase with induction is shown for the wild-type and mutant enhancers by numbers over the solid bars. (**Panels C and D**) Gel mobility shift assays of the enhancer mutants. Gel shift assays were done using the wild-type and mutant enhancer probes in conjunction with induced K562 nuclear extract (**panel C**) or HeLa cell nuclear extract (**panel D**). Bands a and b represent double and single site occupancy as shown in FIGURE 4B.

implicated in inducible gene expression in other systems.[14,29] The fact that gel shift assays demonstrate protein complex formation on the enhancer with extracts from all cell types tested is consistent with the involvement of an interaction of AP-1 with this enhancer. In addition, we have shown that *in vitro*–synthesized c-Jun–c-Fos heterodimer binds either AP-1 site in the enhancer and comigrates with the single-site band seen with HeLa cell nuclear extracts. We also have preliminary data that shows *trans*-activation of the enhancer-globin reporter plasmid in F9 cells with a c-Jun expression vector. This *trans*-activation is increased by induction of c-Fos expression. Despite these data, it is probable that AP-1 is not sufficient for full activity of the enhancer in erythroid cells. The enhancer is much less active in HeLa cells,[17] despite known AP-1 activity in this cell type, suggesting that this enhancer is erythroid specific. Of interest is a recently described erythroid-specific transcription factor designated NF-E2.[30,31] This factor binds the AP-1 consensus sequence and is necessary for inducible expression of the erythroid-specific gene porphobilogen deaminase (PBGD) in MEL cells. We are currently investigating the role of NF-E2 in activating the HS II enhancer. It is likely that the composition of the protein complex that forms on the enhancer differs between cell types and even within a given cell at different stages of development. These changes in complex formation may modulate transcriptional activity of the affected gene.

This is the first description of a discrete element within the β-globin LAR, but it is certain that other critically important elements exist. Although the 46-bp fragment alone confers inducible globin expression in our stable transfection assay, the absolute level of expression is much less than when HS IV and HS III are included in the construct or when the larger HS II fragment is assayed. These findings show that other sequences within HS IV, III or II, which are inactive without the enhancer, can greatly augment transcriptional activity when the minimal enhancer is included in the construct. This activity seems to reside at multiple locations within the LAR and may coincide with redundant sequence motifs. These sequences may function *in vivo* by altering chromatin structure within erythroid cells so as to render the entire domain into an active configuration. Mapping studies may show these elements to be more diffuse than the HS II enhancer. Further dissection of the LAR will be essential to a more complete understanding of this important and complex regulatory region.

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The Dominant Control Region of the Human β-Globin Domain

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INTRODUCTION

The globin genes encode the α- and β-like polypeptides which form the subunits of the oxygen-binding protein hemoglobin. In the early stages of human development, when the embryonic yolk sac is the hematopoietic tissue, the ζ- and ε-globin genes are expressed; this is followed by a switch to the α- and γ-globin genes in the fetal liver and the α- and β-globin genes in adult bone marrow (for review, see Ref. 1). The genes are expressed at exceptionally high levels and give rise to 90% of the total soluble protein in circulating red blood cells.

The β-like globin genes are arranged in the same order as they are expressed during development, i.e., 5′-ε-^{G}γ-^{A}γ-δ-β-3′, over a distance of 55 kb on the short arm of chromosome 11 (FIG. 1; for review see Ref. 1). The DNA sequence analysis has been completed for the entire region, and a comparison with the recently completed sequence data for the rabbit β-globin gene domain shows that the entire loci are homologous, with the exception of repetitive sequence insertions and some duplications and deletions.[2] A large number of structural defects have been documented in and around the β-globin gene (for review, see Refs. 1, 3) which are responsible for a heterogeneous group of genetic diseases collectively known as the β-globin thalassemias. These diseases are not only clinically important, but they also provide natural models for the study of the regulation of globin gene transcription and the mechanism of gene switching during development. Many of the β-thalassemias result from point mutations affecting either the processing or the normal coding capacity of the mRNA (for review, see Ref. 1). More interesting in terms of transcriptional regulation are the promoter mutations and deletions. Many of the δβ-thalassemias and most forms of hereditary persistence of fetal hemoglobin (HPFH) are characterized by deletions of varying size, and some of these are associated with an elevated expression of the γ gene in adult life. Analysis of the positions of these deletions has suggested that they give rise to effects in *cis* that act over considerable distances to influence differential gene expression within the human β-globin domain. In addition, there is the non-deletion type HPFH, which has recently been shown to contain single base-pair changes in the upstream promoter sequences of the γ-globin genes. Interestingly, the level of β-globin expression is reduced concomitantly, which suggests that the genes are coordinately regulated by elements (and factors) in *cis*

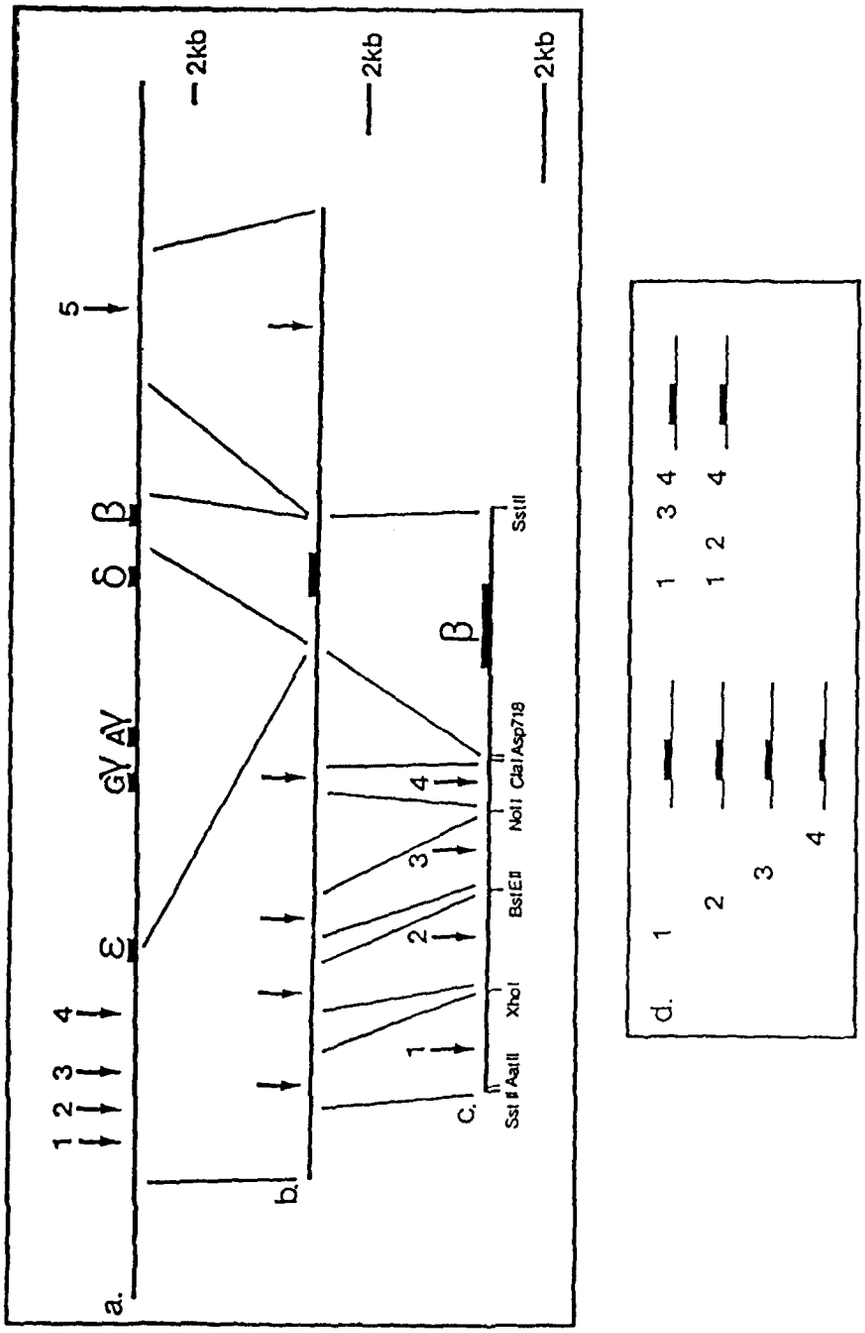

FIGURE 1. *Schematic illustration of the β-globin dominant control region (DCR) constructs. Numbers represent the hypersensitive site(s) present in each construct.* (**a**) The human β-globin locus on chromosome 11. (**b**) The original cosmid β-globin minilocus was derived using the 5′ and 3′ elements illustrated.[11] (**c**) The β-globin microlocus cassette containing the four DNase I–hypersensitive site fragments. The unique restriction enzyme sites flanking each hypersensitive site are illustrated.[14,16] (**d**) Deletion variants of the microlocus microinjected into fertilized mouse eggs to produce *transgenic mice.*

TABLE 1. Expression of DCR Microlocus Deletion Constructs in Transgenic Mice

Construct[a]	Mouse No.	Expression (cpm)[b] Huβ	Mβ	Huβ/Mβ[c]	Copy No.	Huβ/Mβ/Copy No.
Deletion constructs						
HS:1	140	1189	1751	0.68	5	0.14
	163	1471	1466	1.0	6–8	0.15
	174	119	266	0.45	20–25	0.02
HS:2	49	1166	1688	0.69	2	0.34
	77	2596	2674	0.97	3	0.32
	348	1160	1252	1.32	4	0.33
	354	1883	790	2.38	40–50	0.05
HS:3	91	458	1666	0.27	2	0.14
	104	2542	4103	0.62	4	0.16
	94	5344	7128	0.75	5	0.14
	103	8204	3974	2	12–18	0.13
HS:4	208	15	410	0.04	1	0.04
	198	27	833	0.033	2	0.02
	333	223	466	0.48	7	0.06
	335	877	576	1.5	30–40	0.04
	210	1222	668	1.8	>60	0.03
HS:124	188	79	99	0.26	3	0.26
	330	1347	1355	0.99	5	0.25
HS:134	32	100	293	0.34	1	0.34
	29	99	47	2.14	7	0.30
Controls						
HS:1234	55	1377	922	1.49	3	0.49
Hu 11	MEL	271	543	0.5	1	0.50

[a]Hypersensitive site(s) (HS) present in each deletion construct are indicated. Hu 11, human chromosome 11 introduced into cultured erythroid MEL cells.

[b]Expression of the human β-globin (Huβ) transgene in the indicated construct and of the endogenous mouse β-globin (Mβ) gene is shown for each transgenic mouse and for the control MEL cells with human chromosome 11.

[c]Ratio of expression of human transgene and endogenous mouse gene.

(for review, see Ref. 3). The first evidence for the existence of an important control in the flanking region of the globin gene domain was provided by the analysis of human γβ-thalassaemias.[4,5] Patients with heterozygous Dutch γβ-thalassaemia have a deletion that removes 100 kb of DNA, leaving the β-globin gene and the control regions described above intact. However, it abolishes expression of the chromosome bearing the deletion and leaves the gene in an inactive chromatin configuration.[6–8] The wild-type allele on the other chromosome is expressed at normal levels, indicating that there is no shortage of factors.

RESULTS AND DISCUSSION

Candidate sequences for a dominant control region were the erythroid-specific DNase I–hypersensitive sites that map 6–18 kb upstream from the ε-globin gene[9–11]

(FIG. 1). When this region is added to a human β-globin gene construct, it results in very high levels of human β-globin gene expression in transgenic mice (TABLE 1), which are related to the copy number and are independent of the integration site of the transgene.[11] When the same minilocus is transfected directly into erythroid cells (MEL and K562), a similar effect is obtained.[12] The expression level per human β-globin gene is at a similar level to that of the endogenous mouse globin genes and is completely erythroid specific. The presence of the flanking region also results in a dramatic stimulation of transcription from non–erythroid-specific promoters such as that of the thymidine kinase (*tk*) gene. A similar stimulation of an SV40-*neo* gene is seen when it is integrated into the human β-globin locus by homologous recombination.[13] Recloning of the upstream hypersensitive sites on small fragments (6-kb total size) still results in a high level of expression from both the β-globin and *tk-neo* genes.[14] The four upstream hypersensitive sites have been termed the β-globin dominant control region (DCR). The very high transcription levels stimulated by the DCR are independent of the orientation and relative positions of the β-globin gene and the DCR; in this respect, the DCR shows the same properties as a classical enhancer.[15] Deletion of the β-globin gene leaves the *tk-neo* gene fully active. Deletion mapping of the DCR using MEL cells shows that the most important sites in the DCR are the hypersensitive sites 2 and 3 (FIG. 1).[16] Each of these gives an approximately equal contribution to β-globin transcription when they are tested in isolation. When each of the sites is tested in transgenic mice (TABLE 1), site 2 is the most effective site, giving approximately 70% of full expression. Sites 1 and 3 are very similar and have about half the activity of site 2 in transgenic mice. Deletion of single sites results in a DCR with less than full activity and shows that all sites may be required for full activity.[22] Functional mapping of the individual sites 1, 2, and 3 shows that the main activity of each is localized in a 200–300 bp region. When the

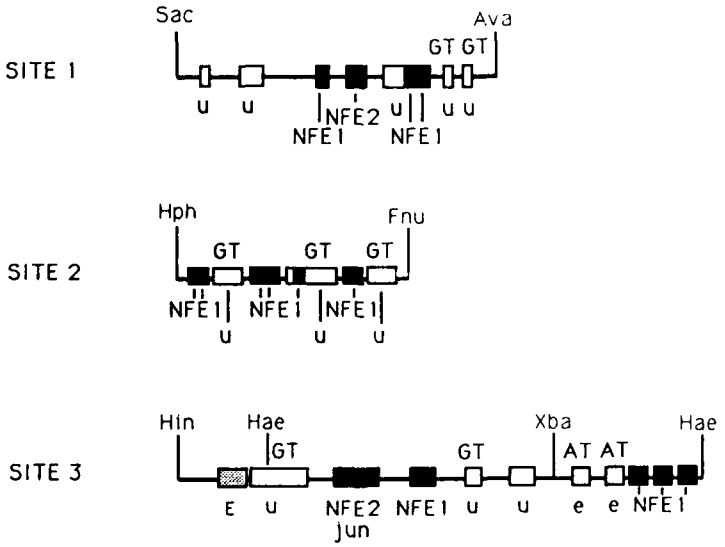

FIGURE 2. Schematic representation of the nuclear factor–binding regions in the minimal DCR element sites 1, 2, and 3. u, ubiquitous factor; e and E, erythroid-specific factors; AT, AT-rich region; GT, the GGTGG consensus.

sites are compared to each other, a number of common features are observed (FIG. 2). Each site contains multiple binding regions for the erythroid-specific transcription factor NF-E1.[17-19] The other striking homology is the occurrence of a GT motif, particularly in site 2. This sequence binds a number of protein factors, including Sp1 and CAC, but it is at present not clear which of the factors are important for function.[23] Site 1 and site 3 each have a binding site for the erythroid-specific factor NF-E2,[20] and in the case of site 3 this binding site has been shown to be essential for function. The presence of this NF-E2 site alone results in a moderate enhancement in MEL cell expression, but it does not function in transgenic mice.[24,25]

We have studied the effect of the complete DCR (or the μDCR[14]) on the expression patterns of the γ- and β-globin genes. This study showed an extension of the expression of the γ-globin gene to the fetal/adult period, in contrast with the

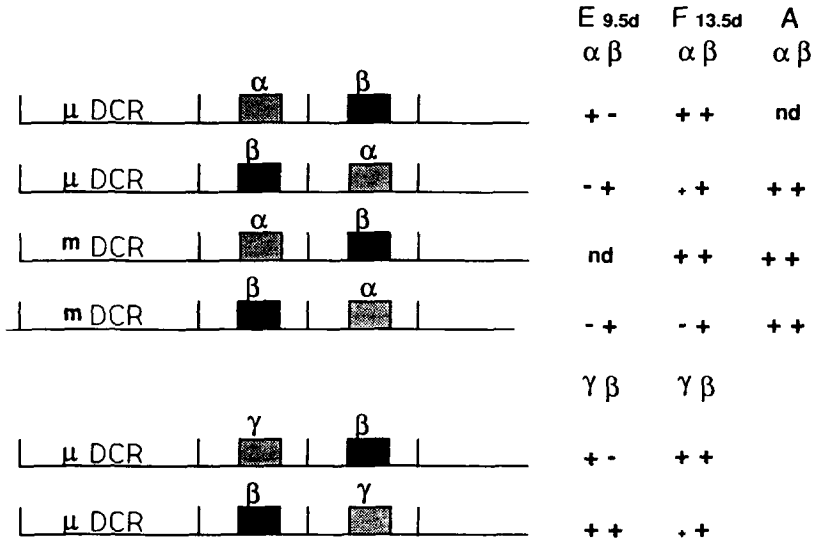

FIGURE 3. Schematic representation of the expression patterns of the α-, γ-, and β-globin genes when tested in different combinations with the DCR. Expression was measured in 9.5-day yolk sac (E9.5d), 13.5-day fetal liver (F13.5d), or adult (A) tissue. Mini-DCR (mDCR) and μDCR indicate the full or shortened version[14] of the dominant control region. nd, not done.

strictly embryonic pattern of expression obtained with the γ-globin gene alone (not shown). Thus, the DCR must influence the developmental expression pattern of the genes. More interestingly, we found that the spatial ordering of multiple genes relative to the DCR further affects the developmental expression pattern of each of the genes. To show this, both a combination of α- and β-genes or of γ- and β-genes was used, and the activity of each gene was determined in the yolk sac (9.5 day) or fetal liver (13.5 day) stage of expression. The results (FIG. 3) show that the developmental expression pattern of the genes is changed depending on their position relative to the DCR. For example, the β-globin gene is expressed (albeit only at 5–10% levels) at the embryonic stage when proximal to the DCR, but it is inactive at this time when the α- or γ-gene is in the proximal position. This indicates that there is a polarity effect from the DCR that influences the developmental expression pattern of the genes, although it is clear that the major developmental

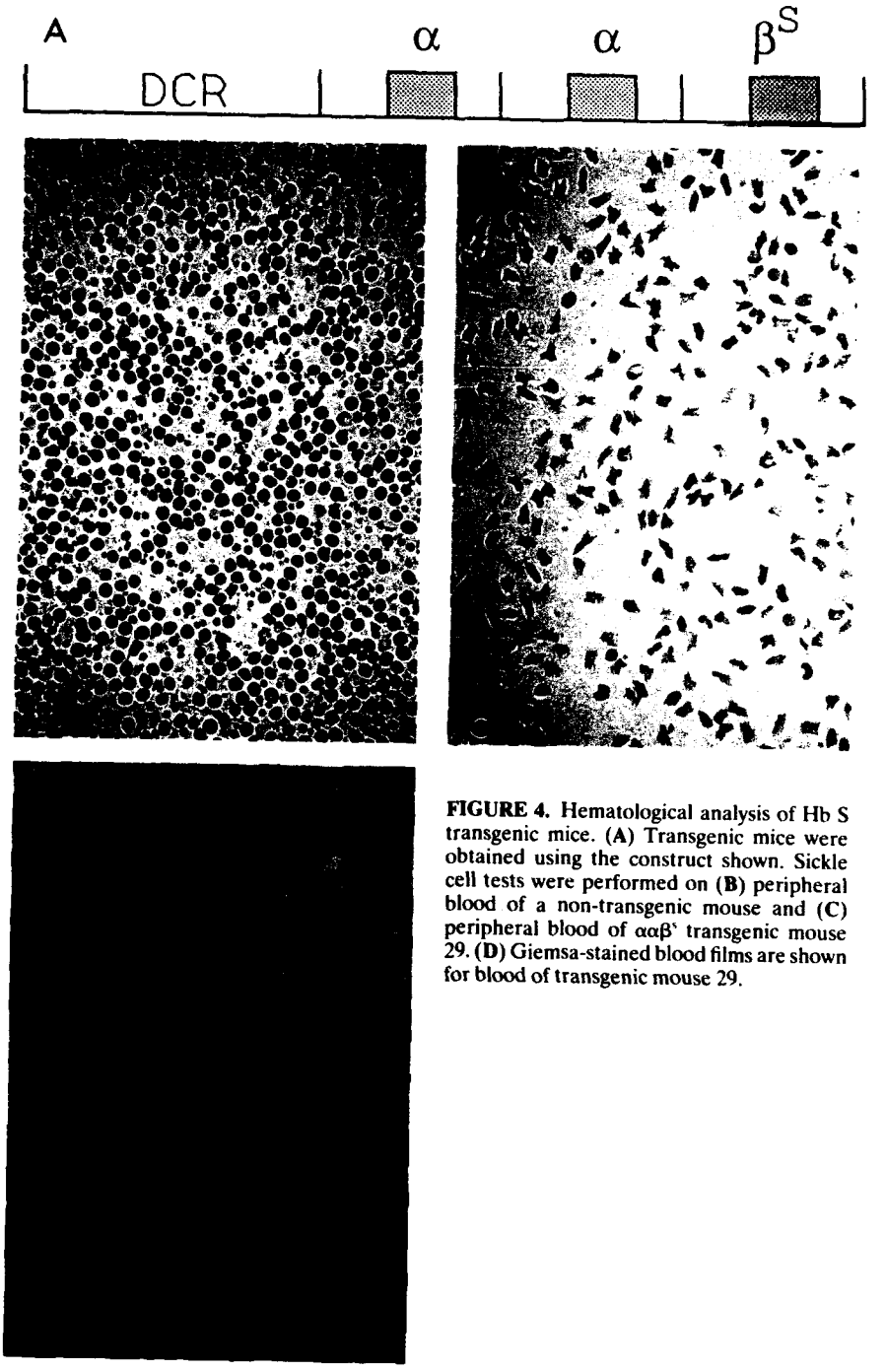

FIGURE 4. Hematological analysis of Hb S transgenic mice. (**A**) Transgenic mice were obtained using the construct shown. Sickle cell tests were performed on (**B**) peripheral blood of a non-transgenic mouse and (**C**) peripheral blood of ααβˢ transgenic mouse 29. (**D**) Giemsa-stained blood films are shown for blood of transgenic mouse 29.

specificity is determined by sequences immediately surrounding the genes (not shown).

We have also used the β-globin DCR to obtain a model for sickle cell anemia in transgenic mice by coupling the DCR to two human α genes and a β^s gene (FIG. 4A). A number of transgenic mice were produced, one of which contained 8–10 copies of the transgene construct.[21] *In vitro* sickle cell tests showed that the red cells of these mice undergo extensive changes in morphology (FIG. 4B, C), and they were shown to contain the typical β^s polymers.[21] The presence of irreversibly sickled cells in blood films (FIG. 4D) indicates that sickling is taking place *in vivo,* although it is at a lower level than in human patients. At present we do not know whether this lower level of sickling is due to the residual expression of mouse hemoglobin or to the different physiology of the mouse. Experiments are in progress to develop a sickle cell disease line for further clinical investigations.

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The Regulation of γ-Globin Gene Expression

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INTRODUCTION

The analysis of globin gene transcription in tissue culture[1-4] and in transgenic mice[5-7] suggests that the expression of β- and γ-globin genes results from the complex interaction of *cis*-acting sequences closely linked to the structural gene with *trans*-acting factors present in erythroid cells. Our research has focused on elucidating the molecular mechanisms involved in γ gene synthesis. The ability to modulate γ-globin gene expression *in vivo* could provide an approach to treatment for patients with β-thalassemia and sickle cell anemia.

The experimental goal is to determine the *cis*-acting sequences and identify the *trans*-acting factors that interact with these sequences to modulate γ-globin gene expression in fetal erythroid cells. In these studies, K562 cells, a human erythroleukemia cell line, were used.[8] These cells express the endogenous ε- and γ-globin genes and do not express the endogenous β-globin gene.[9,10] In addition, transfected ε-globin genes are expressed in these cells, whereas transfected β-globin genes are not.[1,2,11,12] When K562 cells are grown in hemin, an increase in ε- and γ-globin mRNA accumulation, known as induction, is observed.[10,13] Thus, these cells represent a suitable cell line in which to study embryonic and fetal globin gene synthesis.

We have taken several approaches to examine the cellular events involved in γ-globin gene expression in K562 cells in more detail. To position the sequences involved in fetal globin gene synthesis we have (1) made hybrid genes comprised of γ-globin and β-globin gene sequences, (2) introduced these genes in plasmids into K562 cells, and (3) characterized the transcription and induction using fusion gene–specific probes. A more precise localization of regulatory elements, to the level of the nucleotide, has been achieved by analyzing the interaction of protein factors from K562 cells with globin gene sequences using DNase I footprinting[14] and gel mobility shift assays.[15]

EXPRESSION OF FUSION GLOBIN GENES IN K562 CELLS

The primary structure of all β-like globin genes is very similar, and the locations of many restriction sites within the structural genes are shared. We used these sites to generate fragments for our hybrid globin gene constructs. Since the γ-globin gene is expressed and the β-globin gene is not expressed in K562 cells, our hypothesis was that γ-β fusion gene constructs would discriminate between sequences that confer transcriptional activation and sequences that either prevent expression or have no effect on globin gene synthesis. Also, globin–neomycin resistance gene (*neo*^{R}) constructs have provided additional information on the role of the structural globin gene in expression and induction.

The hybrid globin genes were subcloned into the plasmid pSV2NEO[16] and transfected into K562 cells either by calcium phosphate precipitation[17] or by electroporation.[18] The *neo*^{R} gene included on pSV2NEO confers selection, in eukaryotic cells, against the toxic effects of G418, a neomycin analogue. Cells resistant to G418 were selected, and either individual clones or pools of clones were grown in mass culture either in the absence or presence of 20 μ*M* hemin. Total RNA was extracted,[19] and the transcripts were analyzed by either RNase protection[20] or S1 nuclease protection.[21] Occasionally, canonical cap site initiation was established by primer extension.[22]

TABLE 1. Transcription of Fusion Globin Genes in K562 Cells

Fusion Gene	Promoter	IVS-2	3′ β Enhancer	Expressed	Induced
β	β	β	No	No	No
βγ	β	β	No	No	No
βγ2	β	γ	No	Yes	Yes
γβ2	γ	β	No	Yes	No
γβ	γ	γ	Yes	Yes	Yes

The results of our analysis of transcription and induction of fusion globin genes are summarized in TABLES 1 and 2. In this analysis the ability of a particular gene to be expressed and/or induced was correlated with the contribution of γ- or β-globin gene sequences in the promoter and intervening sequence 2 (IVS-2) and with the presence or absence of the β-globin 3′-enhancer element in the construct. Our initial analysis included the five genes in TABLE 1.[23] The β-globin gene containing the β promoter, the β IVS-2, and no β 3′ enhancer was neither expressed nor induced in K562 cells. Similarly, the βγ gene containing only the substitution of the γ 3′ region for the β 3′ region was not expressed or induced. In contrast, βγ2, which contained the β promoter, the γ IVS-2, and no β 3′ enhancer, showed evidence of expression and induction when transcripts were analyzed with a 3′ probe. However, these transcripts were not appropriately initiated, as indicated by primer extension analysis performed to characterize the 5′ end of the mRNA. γβ2, containing the γ promoter, the β IVS-2 and no 3′ enhancer, was expressed but not induced. γβ, containing the γ promoter, the γ IVS-2 and the β 3′ enhancer was expressed and induced in K562 cells.[23]

In order to more precisely define the relationship of the promoter, IVS-2, and β 3′ enhancer to globin gene expression and induction in K562 cells, the fusion genes

TABLE 2. Transcription of Additional Fusion Globin Gene Constructs in K562 Cells

Fusion Gene	Promoter	IVS-2	3′ β Enhancer	Expressed	Induced
$\gamma^P\beta$	γ	β	Yes	Yes	Yes
$\gamma^P\beta\Delta3'$	γ	β	No	Yes	No
$\gamma^P\beta\gamma2\Delta3'$	γ	γ	No	Yes	Yes
γNeo	γ	—	No	Yes	No
γNeo3′β	γ	—	Yes	Yes	Yes

described in TABLE 2 were analyzed.[24] $\gamma^P\beta$, containing the γ promoter, the β IVS-2 and the β 3′ enhancer was both expressed and induced. When the β 3′ enhancer was deleted from this construct to generate $\gamma^P\beta\Delta3'$, this fusion gene retained its ability to be expressed; but no increase in mRNA transcript accumulation was observed in response to hemin induction. The substitution of the γ IVS-2 for the β IVS-2 in $\gamma^P\beta\Delta3'$ to generate $\gamma^P\beta\gamma2\Delta3'$ restored inducible expression to this fusion gene. Finally, with the γNeo fusion genes the interaction of globin structural gene sequences with 5′ promoter elements and 3′ enhancer elements was determined. γNeo, containing the γ promoter, the *neo*^{R} coding sequence and no β 3′ enhancer, was expressed but was not inducible.[25] When the β 3′ enhancer was linked in *cis* on this construct to generate γNeo3′β, inducible expression was observed.[24]

Several conclusions result from the analysis of fusion globin gene expression and induction in K562 cells. First, the γ promoter is required for baseline expression in fetal erythroid cells. No gene containing the β promoter produced correctly initiated transcripts. In fact, only one gene containing the β promoter (βγ2) is either expressed or induced in K562 cells, and this expression is only observed when a 3′ probe is used for the assay. When the transcripts are hybridized with a 5′ probe or are subjected to primer extension analysis, no discrete 5′ end is detected. These data suggest that genes containing the β promoter are not correctly initiated, either because the gene is under negative regulation in K562 cells or because these cells do not contain *trans*-acting factors that are required for β-globin synthesis. In addition, either the γ IVS-2 or the β 3′ enhancer is able to confer inducible expression on genes containing either of these elements linked in *cis*. The molecular mechanism responsible for induction by these elements remains to be elucidated.

TRANS-ACTING FACTORS THAT INTERACT WITH THE γ-PROMOTER

Analysis of fusion globin gene transcription indicated that the γ-globin promoter is important in fetal cell–specific gene expression. In addition, single nucleotide changes between nucleotides −150 and −204 (relative to the cap site) have been reported to cause the non-deletion form of hereditary persistence of fetal hemoglobin (HPFH).[26] Using a combination of methods, including the gel mobility shift assay and DNase I footprinting, we examined the interaction of nuclear factors with this region. A 240-basepair (bp) probe containing sequences between −140 and −382 was used in DNase I footprinting studies. The nucleotide changes associated with HPFH that are found on this fragment include −158, −175, −196, −198, and −202. Although several footprints were detected, our analysis focused on the footprint around the −170 to −194 region, a sequence that contains the −175 mutation involved in one form of HPFH.

Nuclear extracts prepared from K562 cells were compared with those from EL-4 cells, a T lymphocyte cell line.[27] In the −170 to −195 region, a broader footprint was

observed over the entire sequence (−170 to −195) with the K562 extract compared to the EL-4 extract, which protected a smaller footprint (−170 to −188). An *Ava* II–*Hae* III fragment (−161 to −204: γ42) was used in gel mobility shift experiments to characterize the number and size of the nuclear factors that interacted with this region. With HeLa cell extract, a single band of high molecular weight was detected (Band A). K562 cell extract generated three bands. In addition to Band A, two additional bands of faster mobility were observed (Bands B and C). In order to identify the proteins that were binding to the γ42 fragment, competition studies were performed. In these experiments, an excess of unlabeled competitor DNA containing either the octamer binding sequence (which binds to factor OTF-1)[28,29] or one of the sequences found in the β 3′ enhancer[30,31] that bind the erythroid-specific factor (NFE-1, GF-1) was used. This analysis indicated that Band A resulted from the interaction of OTF-1 with γ42 and that both Bands B and C were generated by the interaction of NFE-1 with the probe.

DNase I footprinting with fractionated K562 nuclear extract was used to characterize the binding sites of both OTF-1 and NFE-1. Following ammonium sulfate precipitation, the K562 nuclear extract was fractionated on heparin-Sepharose. The column was eluted with an ammonium sulfate step gradient. By use of the gel mobility shift assay, a sensitive indicator of nuclear proteins present in the eluate, the elution of OTF-1 at 0.2 *M* ammonium sulfate and NFE-1 at 0.4 *M* ammonium sulfate was observed. DNase I footprinting with the fractionated extract positioned the OTF-1 footprint from −172 to −189; the NFE-1 footprint overlapped and was found between −175 and −194 (FIG. 1). To evaluate whether the point mutation at −175 had altered binding for either NFE-1 or OTF-1, competition analysis with oligonucleotides containing either the wild-type octamer binding sequence or the same sequence containing the −175 mutation was used in the gel mobility shift assay. This analysis showed that the −175 mutation eliminated the ability of the oligonucleotide to compete for OTF-1 binding, indicating that the mutant sequence was deficient in its ability to bind this factor. These results suggest that the decreased OTF-1 binding in the presence of the −175 mutation may permit increased NFE-1 binding, resulting in increased γ gene transcription in HPFH.

SUMMARY

In summary, our analysis indicates that important sequences for the proper initiation of fetal gene transcription in fetal cells are located in the γ-globin

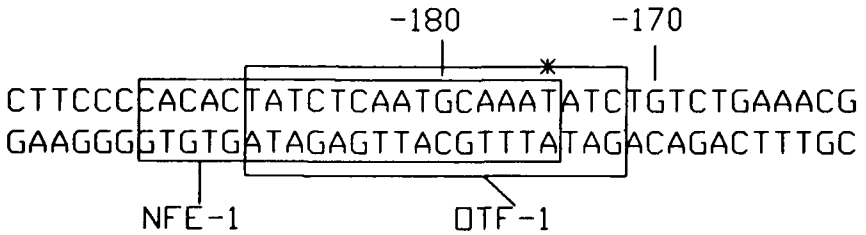

FIGURE 1. Nucleotide sequence of the γ-globin promoter between −161 and −200 relative to the cap site. The mutation at −175 associated with HPFH is indicated by an *asterisk*. The overlapping footprints generated by NFE-1 and OTF-1 binding to this region are delineated by the *boxes* surrounding the nucleotides involved.

promoter. These sequences are sufficient for tissue-specific expression but not induction in K562 cells. Sequences in the γ-globin IVS-2 and the β-globin 3′ enhancer increase γβ and γ-*Neo* transcripts when cells containing these genes undergo erythroid maturation as measured by induction with hemin. The mechanism by which these sequences exert their effect remains to be elucidated.

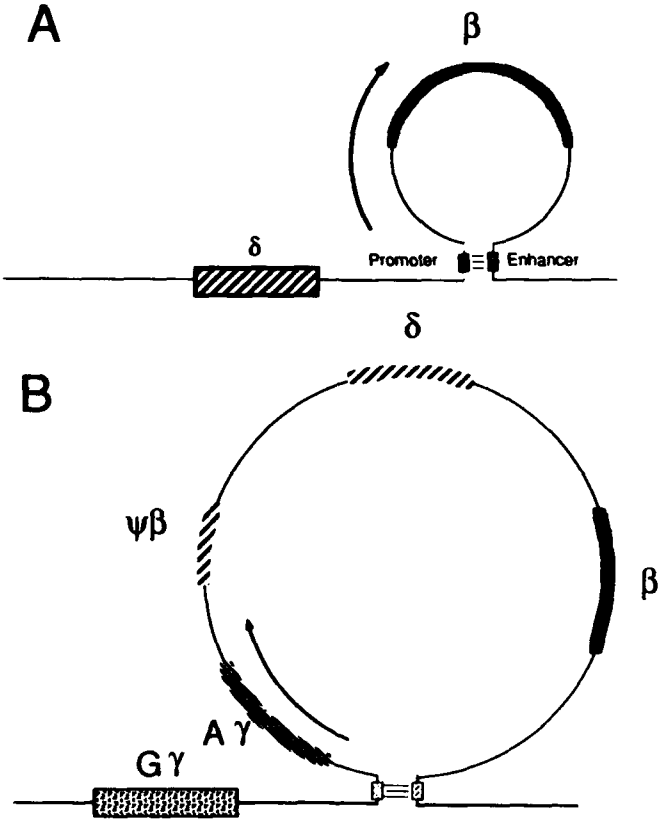

FIGURE 2. Model of developmental regulation of globin gene synthesis by competition for the β enhancer. In this model the β 3′ enhancer is able to influence the expression of both the γ- and β-globin genes. Factor-factor interactions would be one mechanism of stabilizing the chromatin to facilitate these interactions. (**Panel A**) In adult cells, interaction between the β-globin promoter and the β 3′ enhancer would confer synthesis of β-globin mRNA. (**Panel B**) In fetal cells, the β 3′ enhancer would interact with the γ-globin gene promoter, inducing the synthesis of γ-globin mRNA.

Multiple protein factors bind to both the γ promoter and the β 3′ enhancer. Both of these regions contain binding sites for the erythroid-specific factor NFE-1 and the octamer binding factor OTF-1. In the γ upstream region, there may be a competition between OTF-1 binding and NFE-1 binding that affects γ gene regulation.

Our results indicate that the β 3′ enhancer interacts with the γ gene promoter to

permit increased γ gene expression. We have developed a model for globin gene switching that takes into consideration the effect of *cis*-acting sequences on globin gene transcription (FIG. 2). A similar model of hemoglobin switching in chickens has been proposed by Choi and Engel.[32] In our model, competition for the β-globin 3′ enhancer is involved in stage-specific transcriptional activation of γ-globin genes in fetal cells and β-globin genes in adult cells. In adult cells the protein-protein interactions between adult cell–specific factors interacting with the β-globin promoter and erythroid-specific factors interacting with the β 3′ enhancer would activate transcription of the β-globin gene (FIG. 2, panel A). In fetal cells (panel B) protein-protein interactions between fetal cell–specific factors interacting with the γ-globin promoter and erythroid-specific factors interacting with the β 3′ enhancer would activate the transcription of the γ-globin genes.

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Expression of Human Globin Genes in Transgenic Mice Carrying the β-Globin Gene Cluster with a Mutation Causing $^{G}\gamma\beta^{+}$ Hereditary Persistence of Fetal Hemoglobin[a]

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INTRODUCTION

The human β-like globin genes are located on the short arm of chromosome 11 as a cluster of five functional genes in the order 5′-ε-$^{G}\gamma$-$^{A}\gamma$-δ-β-3′.[1] Each globin gene is expressed at a sequential stage of development. The ε-globin gene is expressed in embryonic (yolk sac–derived) erythroid cells, the $^{G}\gamma$- and $^{A}\gamma$-globin genes are expressed in fetal erythroid cells, and the δ- and β-globin genes are expressed primarily in adult erythroid cells.[2] In normal adults, the level of fetal hemoglobin is less than 1% of the total hemoglobin.[2] In a heterozygous non-deletion form of hereditary persistence of fetal hemoglobin (HPFH) called $^{G}\gamma\beta^{+}$ HPFH, the level of fetal hemoglobin ranges from 15% to 25% of the total hemoglobin and is composed predominantly of $^{G}\gamma$ chains.[3] A single base substitution (C → G) was identified 202

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base-pairs 5′ to the cap site (position −202) of the $^{G}\gamma$-globin gene in an affected individual,[4] and the presence of this mutation was shown to be closely associated with the $^{G}\gamma$ HPFH phenotype in different individuals.[5]

The generation of transgenic mice has been a useful tool for the study of the developmental regulation of human globin genes. When cloned human β-globin and γ-globin genes containing a limited amount of 5′- and 3′-flanking DNA are introduced into mouse oocytes and transgenic lines are established, these individual genes are regulated during murine development in a manner similar to that of their murine homologs. The human β-globin gene is expressed at the fetal liver stage as well as the adult stage of erythropoiesis in a manner analogous to that of the adult murine β^{major}-globin gene,[6-9] while the γ-globin gene is expressed only at the embryonic (yolk sac) stage of erythropoiesis in a manner analogous to that of the embryonic murine β^{h1}-globin gene.[7,10,11] In this paper, we describe expression studies of human globin genes in transgenic mice which carry the 40-kilobase (kb) *Kpn* I fragment of the human β-like globin gene cluster from an individual with the −202 $^{G}\gamma\beta^{+}$ HPFH mutation. The pattern of expression of the human β-, $^{G}\gamma$- and $^{A}\gamma$-globin genes differed from that of their corresponding murine homologs. The $^{G}\gamma$-globin gene with the −202 HPFH mutation was expressed at all developmental stages. The normal β-globin gene was expressed in adult erythroid cells but was virtually inactive in fetal erythroid cells, whereas the normal $^{A}\gamma$-globin gene was expressed beyond the embryonic (yolk sac) stage into the fetal stage of development and then became inactive in adult erythroid cells.

MATERIALS AND METHODS

Production of Transgenic Mice

The 40-kb *Kpn* I fragment containing the −202 $^{G}\gamma$ HPFH mutation was purified from a cosmid clone[4] by agarose gel electrophoresis or sucrose gradient centrifugation, followed by centrifugation on a CsCl gradient and dialysis.[12,13] The DNA was injected into the pronuclei of (C57BL/6J × SJL/J)F2 or (C57BL/6J × DBA/2J)F2 fertilized eggs as described previously.[12,13] Transgenic mice were identified by Southern blot analysis of tail DNA. To generate transgenic embryos and fetuses, heterozygous transgenic males were mated with normal C57BL/6J females. The day that the mating plug was observed was designated day 0; pregnant females were sacrificed on the indicated gestation day. Transgenic embryos in each litter were identified by dot-blot analysis of DNA prepared from the unused carcass.

Preparation of RNA

Adult mice were made anemic by three injections (at 12-h intervals) of a solution of 0.4% phenylhydrazine[11] and sacrificed 5 days after the first injection. Blood was collected by retro-orbital bleeding into phosphate-buffered saline containing 10 U/ml of heparin (PBS-heparin), and cells were recovered by centrifugation. Transgenic embryos were bled from the umbilical cord into PBS-heparin. Blood cells or whole tissues were washed in PBS-heparin, and total RNA was isolated by homogenization in 4 *M* guanidinium isothiocyanate followed by centrifugation through CsCl.[14]

RNase Protection Assay

The labeled antisense RNA probes used to measure $^{G}\gamma$ and $^{A}\gamma$ RNA were synthesized from the *Eco*R I–*Sau*3A fragments of the third exon of the $^{G}\gamma$ and $^{A}\gamma$ genes, where four consecutive base differences occur between the $^{G}\gamma$ and $^{A}\gamma$ sequences starting at a point three bases 3′ to the termination codon.[15] The labeled antisense RNA probe used to measure human β-globin mRNA was synthesized from an *Eco*R I–*Pst* I fragment spanning the poly(A) addition site. Each probe (1×10^5 cpm per assay) was hybridized with 10 μg of total cellular RNA at 50°C for 18 h in 30 μl of a solution containing 40 m*M* PIPES, pH 6.5, 0.4 *M* NaCl, 1 m*M* EDTA, and 80% formamide. The RNA samples were digested with RNase A (40 μg/ml) and RNase T1 (2 μg/ml) for 30 min at 37°C and then digested with proteinase K (50 μg/ml) for 15 min at 37°C. The protected fragments were analyzed by electrophoresis in a 7.5% polyacrylamide–7 *M* urea gel.

Northern Blot Analysis

Northern blot analysis was performed using the following 19-mer oligonucleotide probes specific for the RNA to be analyzed: 5′-ACAGCAAGAAAGCGAGCTT-3′ (human β), 5′-CATCATGGGCAGTGAGCTC-3′ (human $^{G}\gamma$), 5′-AGAGCAG-GAAAGGGGGTTT-3′ (mouse β^{major}), and 5′-GTGTACTGGAATGGAGTTT-3′ (mouse β^{h1}). These correspond to sequences that start at the second nucleotide of the termination codon of these four globin mRNAs. The oligonucleotides were labeled with [γ-^{32}P]ATP by polynucleotide kinase. Total RNA from mouse tissues was fractionated by formaldehyde gel electrophoresis[16] and transferred to GeneScreen Plus membranes (New England Nuclear). Filters were hybridized with the appropriate oligonucleotide probe (2×10^5 cpm/ml) at 42°C in a solution containing 5× SSC, 0.5% SDS, 5× Denhardt's solution, 50 m*M* sodium phosphate, pH 7.0, and 200 μg/ml of salmon sperm DNA. Following hybridization, the filters were washed twice for 5 min at room temperature in a solution containing 2× SSC and 0.5% SDS, followed by a 10-min wash in the same solution at 45°C.

RESULTS

Structural Analysis of the Human β-like Globin Gene Cluster in Transgenic Mice

In order to determine the pattern of integration of the 40-kb fragment, tail-skin DNA from individual transgenic mice was analyzed by Southern blotting. The −202 mutation of the $^{G}\gamma$-globin gene abolishes a normal *Apa* I site in the 40-kb *Kpn* I fragment.[5] Therefore, digestion with *Apa* I allows direct confirmation of the presence of the mutation in the insert. As shown in FIGURE 1, fragments of 21.7 kb and 18.2 kb were detected in *Apa* I digests of DNA from four transgenic mouse lines following hybridization with a $^{G}\gamma$ cDNA probe. Fragments of the expected normal sizes were detected in the *Eco*R I digests. These results demonstrate that all the transgenic mice have intact 40-kb inserts that are integrated in tandem arrays. The number of copies of the insert per cell in transgenic lines 3, 4, 6, and 10 was estimated to be approximately 60, 20, 10 and 5, respectively.

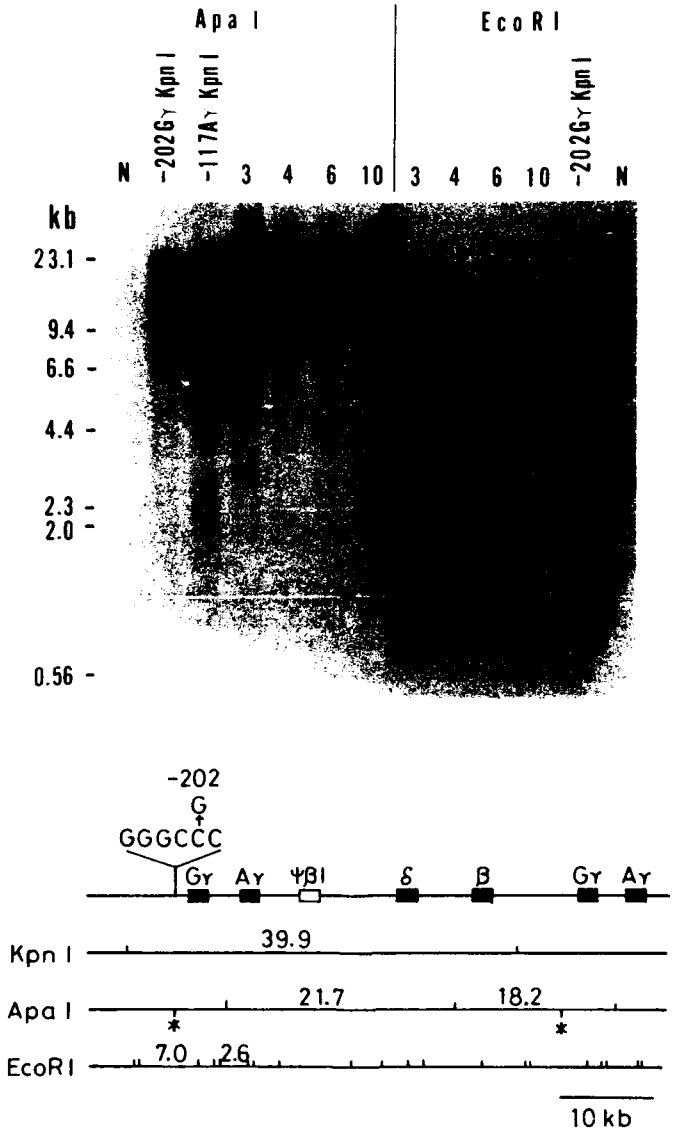

FIGURE 1. Structural analysis of the human β-globin gene cluster in transgenic mice. (**Upper panel**) 10 μg of tail DNA was digested with *Apa* I (**left**) or *Eco*R I (**right**), electrophoresed in a 0.5% agarose gel, transferred to a nylon membrane, and hybridized to a $^{G}\gamma$-globin cDNA probe. The number of each transgenic line is indicated above the respective lanes. N, tail DNA from non-transgenic control mice. 10 ng each of the microinjected *Kpn* I fragment (−202Gγ Kpn I) and the 40-kb *Kpn* I fragment from a Greek-type $^{A}\gamma$ HPFH gene containing wild-type $^{G}\gamma$-, δ- and β-globin genes and the $^{A}\gamma$-globin gene with a G → A mutation at position −117 (−117Aγ Kpn I) were analyzed as positive controls. The locations of DNA molecular weight standards (*Hin*d III fragments of λ DNA) are shown to the *left* of the blot. (**Lower panel**) The diagram shows the 40-kb *Kpn* I fragment aligned in a head-to-tail tandem array and the resulting map of restriction sites. The *Apa* I site is abolished by the −202 C → G mutation, as indicated by the *asterisks*.

Expression of Human Globin Genes in Adult Mice

Expression of human globin mRNA in adult tissues of two transgenic mouse lines (lines 3 and 6) was examined by the RNase protection assay (FIG. 2). Adult mice were made anemic by phenylhydrazine treatment. Hemin-induced K562 cells[17] and cytosine arabinoside–induced KMOE cells[18] were used as positive controls for γ-globin mRNA and β-globin mRNA, respectively. The specificity of the $^{G}\gamma$ and $^{A}\gamma$ probes was determined by the protection patterns observed with $^{G}\gamma$ and $^{A}\gamma$ control RNAs, which were synthesized *in vitro* using T7 RNA polymerase and the *Eco*R I–*Sau*3A fragments cloned in the plasmid vector pGEM. The $^{A}\gamma$ probe was derived from the cosmid clone containing the −202 $^{G}\gamma\beta^{+}$ HPFH 40-kb *Kpn* I fragment, which has a deletion of 6 base-pairs 5′ to the poly(A) addition signal, AATAAA, of the $^{A}\gamma$-globin gene. Therefore, a fragment of 134 bases (instead of the expected 161 bases, as obtained with synthetic $^{A}\gamma$ RNA) is protected when this $^{A}\gamma$ probe is hybridized to $^{A}\gamma$-globin mRNA from K562 cells (FIG. 2B). The expected fragment of 161 bases was protected when the $^{G}\gamma$ probe was hybridized to various RNAs (FIG. 2A). The $^{G}\gamma$ probe yielded a strong positive hybridization signal with blood and spleen (but not kidney or liver) RNA from adult anemic transgenic mice, whereas no hybridization signal was obtained from any of the mouse tissues with the $^{A}\gamma$ probe.

FIGURE 3 shows the results of the RNase protection assays using the human β-globin probe. The specific 212-bp fragment protected by the human β-globin probe was detected in adult blood and spleen RNA but not in liver and kidney RNA from either line 3 or line 6 transgenic mice. The protected fragment was not detected in spleen RNA from a non-transgenic anemic mouse which was producing large amounts of endogenous mouse β^{major}-globin mRNA. These results show that the human $^{G}\gamma$- and β-globin genes are expressed specifically in erythroid tissues of anemic adult transgenic mice. The level of human β-globin mRNA in the line 3 transgenic mice was approximately 1.0% of that of endogenous mouse β^{major}-globin mRNA, and the level of $^{G}\gamma$-globin mRNA was approximately 20% of that of human β-globin mRNA. These levels were estimated by Northern blot analysis of the RNA using the specific 19-mer oligonucleotide probes listed in MATERIALS AND METHODS (data not shown).

Expression of Human Globin Genes during Mouse Development

We also analyzed the pattern of expression of the individual human globin genes during development in the transgenic mice. RNA samples were isolated from erythroid tissues of mouse embryos at day 11 and day 14 of gestation, and these were analyzed for the presence of the human globin mRNAs. FIGURE 4 shows the expression patterns of $^{G}\gamma$- and $^{A}\gamma$-globin genes during development of line 3 transgenic mice. The $^{G}\gamma$-globin mRNA was detected in all of the RNA samples tested, including day 11 yolk sac and blood, day 14 liver and blood, and adult blood. The $^{A}\gamma$-globin mRNA was detected, but at significantly lower levels, in embryonic (day 11) and fetal (day 14) erythroid tissues. In fetal (day 14) liver RNA, the level of $^{A}\gamma$-globin mRNA was 20-fold lower than that of $^{G}\gamma$-globin mRNA. In contrast to the case of $^{G}\gamma$-globin mRNA, $^{A}\gamma$-globin mRNA was virtually absent in adult blood cell RNA.

FIGURE 5A shows RNase protection assays obtained with RNA samples from line 3 transgenic mice using the human β-globin probe. Human β-globin mRNA was not detected in RNA from day 11 blood and yolk sac or from day 14 fetal blood. Barely detectable levels of human β-globin mRNA were observed in RNA from day

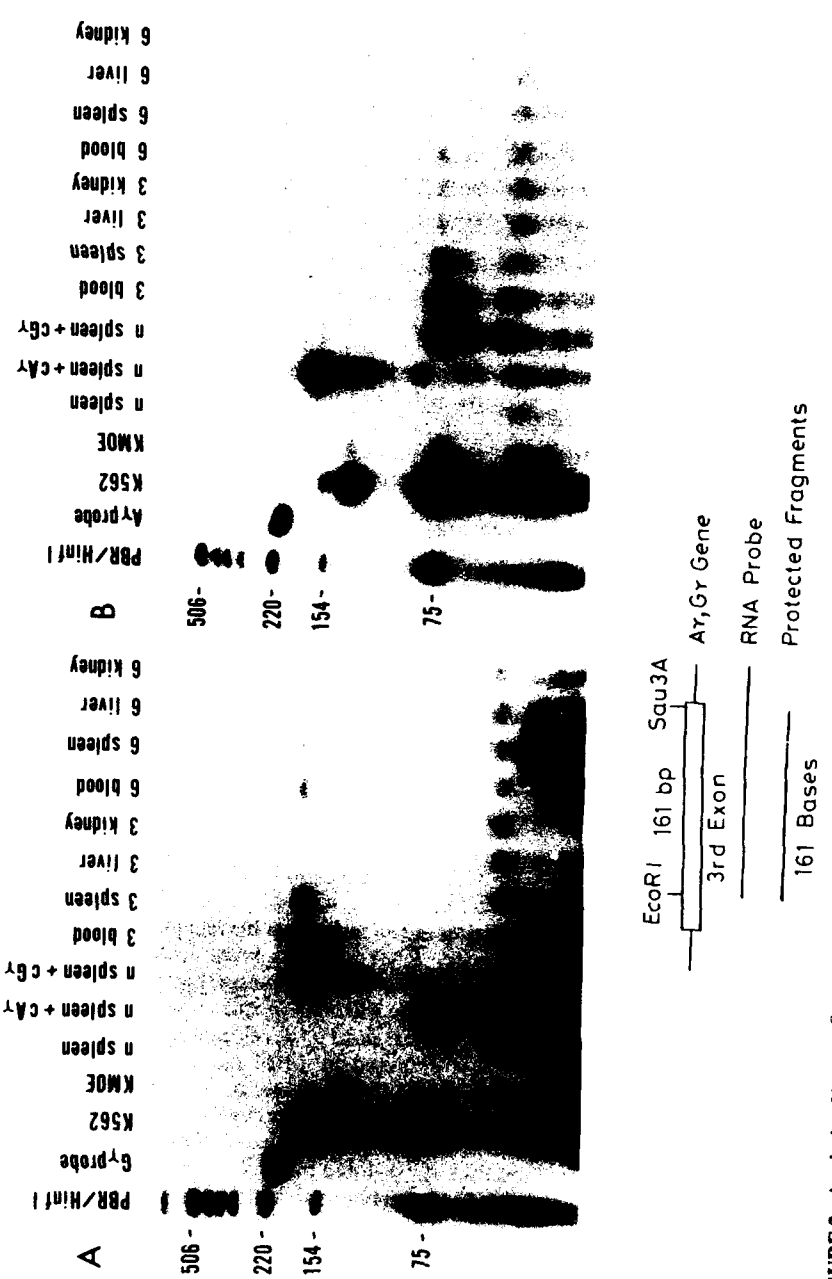

FIGURE 2. Analysis of human $^{G}\gamma$- and $^{A}\gamma$-globin gene expression in adult transgenic mice. Total RNA was isolated from tissues of adult anemic mice. 10 μg of total RNA was hybridized to the $^{G}\gamma$-globin (**panel A**) or $^{A}\gamma$-globin (**panel B**) antisense RNA probes illustrated below the autoradiographs. The protected RNAs were analyzed by 7 *M* urea–7.5% polyacrylamide gel electrophoresis. The specificity of the $^{G}\gamma$ and $^{A}\gamma$ probes was tested with 20 pg of synthetic control $^{G}\gamma$ (cGγ) or control $^{A}\gamma$ (cAγ) RNA mixed with 10 μg of spleen RNA from non-transgenic mice (n spleen). The autoradiographs were exposed for 3 days. The numbers (3, 6) designate the transgenic line from which the indicated tissue RNA was isolated.

14 fetal liver. FIGURE 5B shows the expression patterns of endogenous adult mouse β^{major}- and embryonic β^{h1}-globin mRNAs in the same RNA samples, analyzed by Northern blot using specific 19-mer oligonucleotide probes. The mouse β^{major}-globin mRNA was present at approximately equivalent levels in RNA from fetal liver, fetal

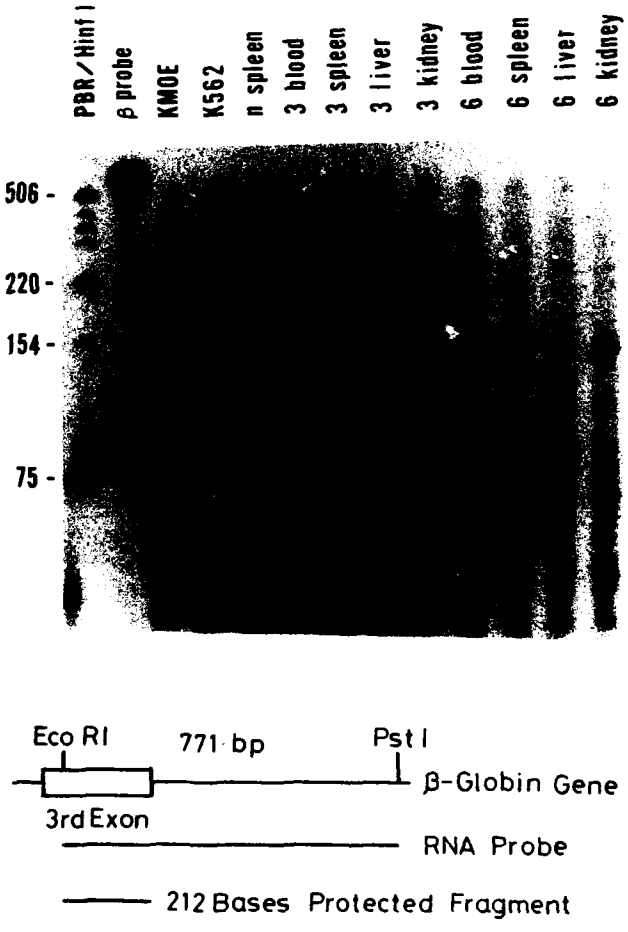

FIGURE 3. Analysis of the expression of the human β-globin gene in adult transgenic mice. The same RNA samples as in FIGURE 2 were analyzed. 10 μg of total RNA was hybridized to the β-globin antisense RNA probe illustrated below the autoradiograph. The protected RNAs were analyzed by 7 *M* urea–7.5% polyacrylamide gel electrophoresis. The autoradiograph was exposed for 2 days.

blood and adult blood, whereas β^{h1}-globin mRNA was detected only in RNA from day 11 blood and yolk sac. It is noteworthy that the same fetal liver RNA sample that contained high levels of mouse β^{major} mRNA yielded barely detectable levels of human β-globin mRNA. This same RNA sample essentially lacked mouse embryonic

β^{hl} mRNA yet contained significant levels of mRNA from the non-mutated $^{A}\gamma$-globin gene.

DISCUSSION

We have analyzed the expression of human globin genes in transgenic mice carrying the 40-kb *Kpn* I fragment from the human β-like globin gene locus of an individual with $^{G}\gamma\beta^{+}$ HPFH. In addition to the normal β-globin human gene, the $^{G}\gamma$-globin gene which carries the −202 point mutation was expressed in erythroid tissues of anemic adult transgenic mice. The expression level of the $^{G}\gamma$-globin gene was approximately 20% of that of the β-globin gene, which is very similar to the level

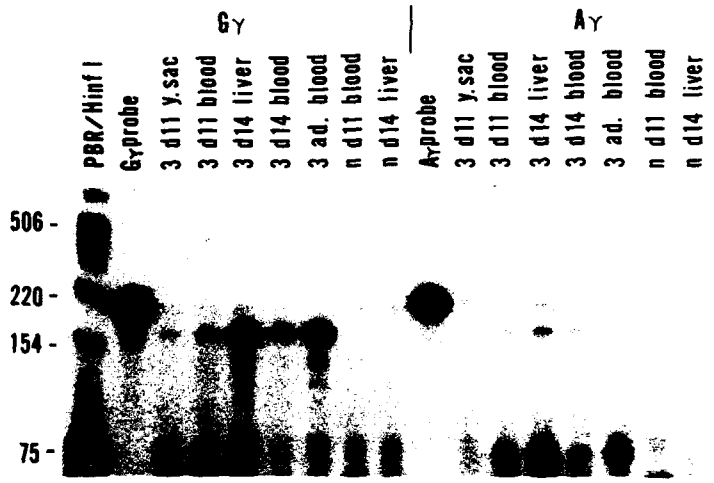

FIGURE 4. Analysis of $^{G}\gamma$- and $^{A}\gamma$-globin gene expression during development in transgenic mice. Total RNA was isolated from day 11 (d11) yolk sac (y. sac) and blood, day 14 (d14) liver and blood, and adult (ad.) blood of line 3 transgenic mice (3) or non-transgenic mice (n). RNase protection assays were performed with the antisense $^{G}\gamma$- or $^{A}\gamma$-globin RNA probes as described in the legend to FIGURE 2. The autoradiograph was exposed for 7 days.

of Hb F in adult red cells of individuals with $^{G}\gamma\beta^{+}$ HPFH. These results suggest that the −202 mutation might contribute to the unexpected expression of the $^{G}\gamma$-globin gene in adult transgenic mice. However, it should be noted that enhancer elements in the third exon and 3′ flanking region of the human β-globin gene[19-23] are now located approximately 10 kb upstream of the $^{G}\gamma$-globin gene in the head-to-tail tandem repeat of the 40-kb insert in the transgenic mice (FIG. 6). The β-globin enhancers are capable of activating $^{A}\gamma$- and $^{G}\gamma$-globin promoters in fetal liver or adult erythroid tissues of transgenic mice when located 0.2 to 2.5 kb from these promoters.[11,20-22] In addition to the −202 mutation, the β-globin enhancers may also contribute to the deregulated expression of the human $^{G}\gamma$-globin gene in adult erythroid cells of the transgenic mice.

It has been previously reported that in transgenic mice, individual cloned human

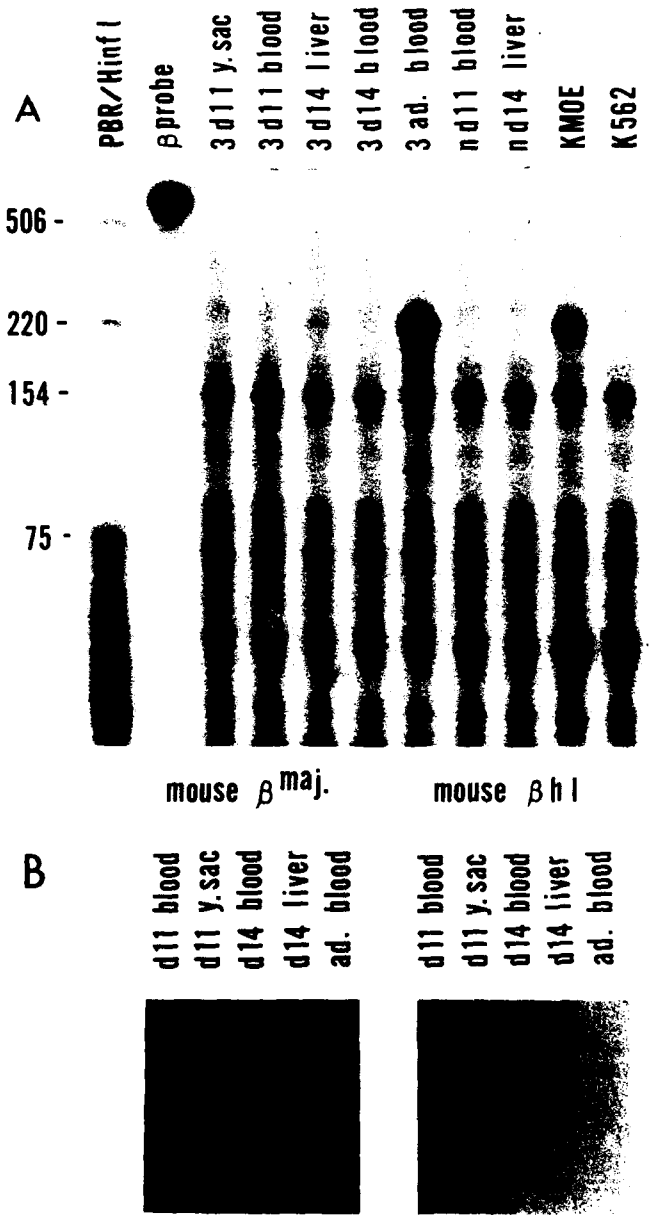

FIGURE 5. Analysis of human β-globin and endogenous mouse β^{major}- and β^{hl}-globin gene expression during development in transgenic mice. The same RNA samples as in FIGURE 4 were analyzed. (**Panel A**) RNase protection assays were performed as described in FIGURE 3 using the human β-globin antisense RNA probe. (**Panel B**) Total RNA (10 μg) was electrophoresed in formaldehyde gels, transferred to nylon membranes, and hybridized to gene-specific 19-mer oligonucleotide probes (see MATERIALS AND METHODS). The autoradiographs in (**A**) and (**B**) were exposed for 3 days and 6 h, respectively. β^{maj}, β^{major}.

β- and γ-globin genes are expressed during development in a manner similar to that of their murine homologs, β^{major} and β^{h1}, respectively.[6-11] However, our results, using the 40-kb *Kpn* I fragment from the human β-globin gene locus, demonstrate a pattern of expression of the normal human $^{A}\gamma$- and β-globin genes that is different from that of their murine homologs. Although the endogenous mouse adult β^{major}-globin gene was fully active in both fetal liver and adult erythroid cells, the human β-globin gene was virtually inactive in fetal liver. Furthermore, whereas endogenous mouse embryonic β^{h1}-globin mRNA was virtually absent in fetal liver RNA, human $^{A}\gamma$-globin mRNA was readily detectable in the same RNA sample. It is noteworthy that the patterns of developmental stage–specific expression of the human β- and $^{A}\gamma$-globin genes in the 40-kb *Kpn* I fragment of our transgenic mice are similar to those observed during human development. The differences in the developmental pattern of expression between individual cloned human genes and those in the 40-kb *Kpn* I fragment of the β-globin gene cluster suggest that the overall organization of the globin gene cluster, including the presence of intergenic DNA, is an important determinant for the stage-specific expression of the human β-like globin genes during development.

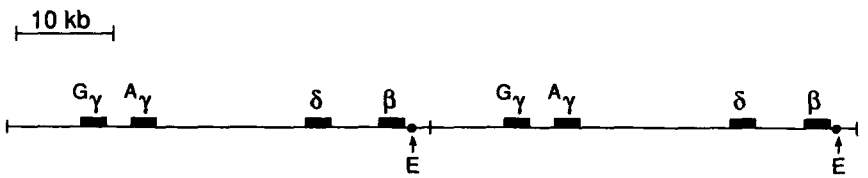

FIGURE 6. Diagram showing the relationship between the β-globin gene enhancer (E) and the $^{G}\gamma$-globin gene promoter in the tandemly repeated 40-kb transgene.

SUMMARY

We have introduced into the mouse germ line the 40-kilobase (kb) *Kpn* I fragment containing the β-globin gene cluster from an individual with a non-deletion form of hereditary persistence of fetal hemoglobin (HPFH) believed to be due to a point mutation at position −202 of the $^{G}\gamma$-globin gene. The $^{G}\gamma$-globin gene, as well as the β-globin gene, was expressed in adult erythroid tissues of the resulting transgenic mice. The level of expression of the $^{G}\gamma$-globin gene was about 20% of that of the β-globin gene. Others have previously shown that cloned individual normal human β- and γ-globin genes containing a limited amount of 5′- and 3′-flanking DNA are expressed in a manner similar to that of their corresponding murine homologs during development in transgenic mice. In contrast, we have observed that the pattern of expression of the normal (non-mutated) $^{A}\gamma$- and β-globin genes in the 40-kb insert was different from that of their corresponding murine homologs. The β-globin gene remained inactive at the fetal stage, whereas the normal $^{A}\gamma$-globin gene was expressed beyond the embryonic (yolk sac) stage into the fetal stage of development and then became inactive in adult erythroid cells. The pattern of expression of the human globin transgenes during mouse development resembles that observed during human development. These results suggest that the gross organization of the human β-like globin gene cluster is important for stage-specific expression of each human globin gene during development.

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Pharmacologic Manipulation of Fetal Hemoglobin in the Hemoglobinopathies[a]

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The regulation of fetal hemoglobin (Hb F) synthesis in the hemoglobinopathies continues to be a subject of great theoretical and practical significance, particularly because high levels of fetal hemoglobin synthesis clearly ameliorate the symptoms of sickle cell anemia and β-thalassemia. In high concentrations, Hb F inhibits the rate and extent of sickling in sickle cells, while in β-thalassemia, γ-chain production leads both to accumulation of hemoglobin in the thalassemic red cell and to a decrease in the accumulation of unmatched α chains, which cause ineffective erythropoiesis. Though the extent of Hb F production in both sickle cell anemia and thalassemia is highly variable, it is clearly inherited; and certain point mutations in γ-globin gene promoters as well as deletions in the γδβ complex are regularly associated with enhanced γ-globin production in β^S and β^{thal} heterozygotes, as well as in homozygotes.[1] In other mutations within the promoter region—such as the −158 C→T substitution in the $^G\gamma$ promoter region seen commonly in individuals from Senegal, the eastern oases of Saudi Arabia,[2] and the Orissa province of India—the effects of the mutation on circulating Hb F are less predictable. Indeed, the Hb F concentrations in peripheral blood samples of β^S heterozygotes also heterozygous for the −158 C→T substitution in the $^G\gamma$ gene are only slightly above normal and are variable.[3] FIGURE 1 demonstrates the effects of the combination of β^S and −158 C→T on the production of Hb F in peripheral blood BFU-E–derived erythroblasts. Note that there is little or no influence of the −158 substitution on Hb F production in the absence of a β^S substitution, whereas in $\beta^A\beta^S$ (AS) heterozygotes, there is a linear increase in Hb F production with increasing dose of the −158 C→T substitution. This effect is markedly enhanced by homozygosity for β^S (SS). Thus, the β^S substitution and the −158 C→T substitution exert a cooperative effect on $^G\gamma$ chain accumulation in BFU-E–derived erythroid cells. The nature of that cooperative effect is not known.

That the Hb F program expressed in BFU-E–derived cells strongly influences the circulating Hb F concentration in Saudi Arabian sickle cell anemia patients with variable doses of the −158 C→T substitution in the $^G\gamma$ promoter is demonstrated in FIGURE 2, which shows that 65% of the variance of peripheral blood Hb F concentration can be explained by the variance of the Hb F program in BFU-E–derived cells. The remainder of the variance of peripheral blood Hb F concentration is likely to be ascribed to the variance in selective survival of Hb F–rich cells.

A recent experiment performed by Dr. Antonio Cao and his associates has shown how dramatically the intrinsic Hb F program in patients with thalassemia influences Hb F production. Sardinian patients with Cooley's anemia are almost always homozygous for one mutation.[4] Heterozygotes for that mutation have variable percentages of Hb F in their peripheral blood, which range generally from approximately 0.5 to 5%, and the value is independent of age or sex. Recently, Dr. Cao has

[a]Supported by grants from the National Institutes of Health and the Dyson Foundation.

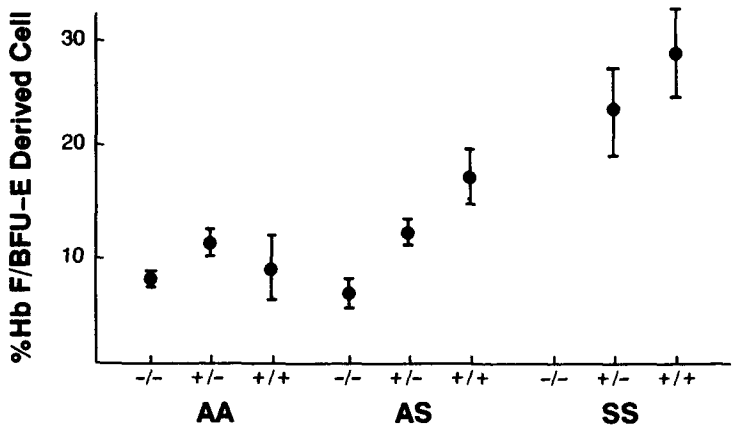

FIGURE 1. The percent of Hb F in peripheral blood BFU-E–derived erythroid cells in Saudi Arabian patients with normal adult Hb (AA), sickle cell trait (AS), or sickle cell anemia (SS) with C (−) or T (+) at −158 in the $^{G}\gamma$ promoter region. Values shown are means ± SE. (From Miller *et al.*[3a] Reprinted with permission.)

had an opportunity to study the Hb F concentration in the peripheral blood of sibling donors of marrow to patients undergoing bone marrow transplantation for homozygous Cooley's anemia and the Hb F concentration in the blood of the sibling recipients two months after transplant. Normal donors have a low percentage of Hb F in their blood, and there is only a small increase in peripheral Hb F in the blood

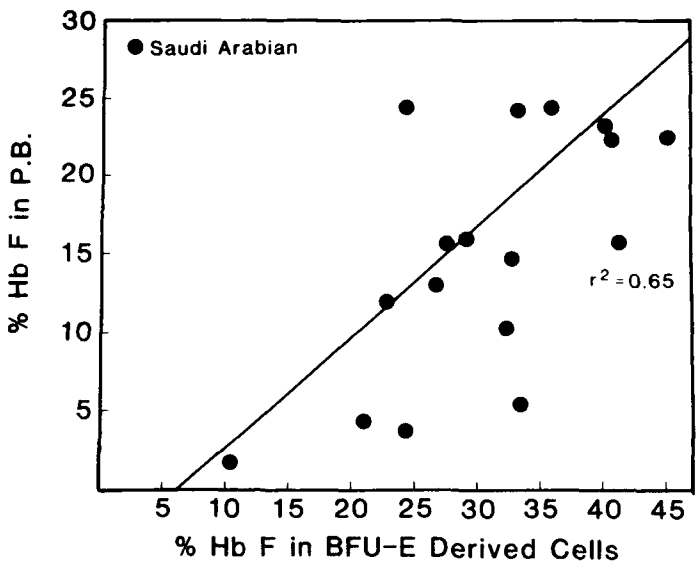

FIGURE 2. The relationship of the percent of Hb F in peripheral blood (P.B.) BFU-E–derived erythroid cells to the percent of Hb F in peripheral blood in Saudi Arabians from the Eastern oases. (From Miller *et al.*[3] Reprinted with permission from *Blood.*)

of recipients of their marrow two months after bone marrow transplantation. On the other hand, donors who are heterozygous for the Sardinian type of β-thalassemia have the expected low, albeit broad, range of Hb F production in their blood prior to transplantation. Two months after transplantation of their marrow to their homozygous siblings, a sharp rise in the Hb F concentration in the blood of the recipients is observed, a rise that correlates extremely well with the original Hb F content in the donor. These findings demonstrate that the Hb F program in the recipient is recapitulated but uniformly stimulated by the stress erythropoiesis associated with marrow transplantation. This result shows that in individuals with thalassemia, the Hb F program is plastic, lending encouragement to the idea that exogenous factors may be found that will stimulate production in homozygotes without transplantation.

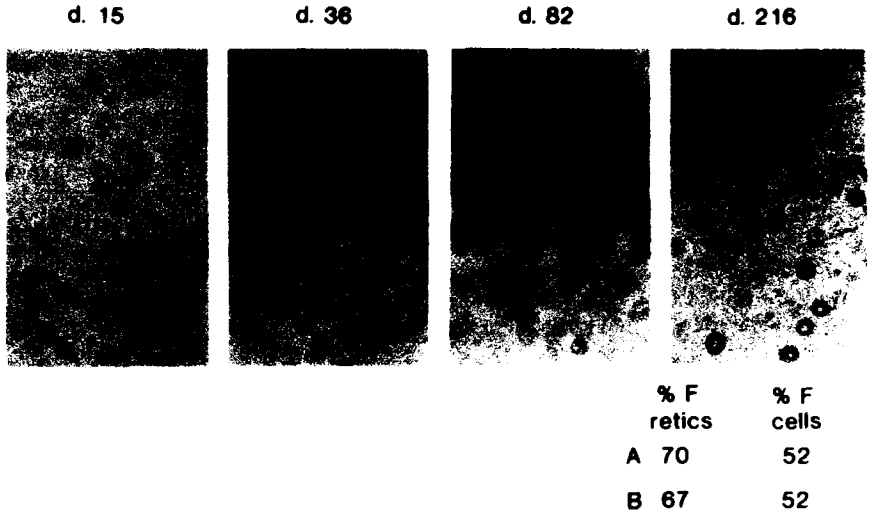

FIGURE 3. Acid elution test of Hb F content in the peripheral blood red cells of cynomolgus monkeys treated for up to 216 days with pulse doses of hydroxyurea. The percent of F reticulocytes and the percent of F cells at the end of treatment were kindly measured by Dr. George Dover. Data derived from the studies of Letvin *et al.*[9]

One of the first exogenous substances that has been found to stimulate Hb F production is the family of S-phase–specific agents, such as hydroxyurea.[5] Stimulated by the findings of DeSimone and co-workers,[6] Ley and co-workers,[7] and Charache and co-workers[8] with respect to 5-azacytidine and its influence on γ-globin production, Letvin and co-workers examined hydroxyurea and its influence on γ-globin production in simians.[9] Their results were striking. Cynomolgus monkeys were bled to simulate hemolytic anemia and then treated with pulse doses of hydroxyurea. There was a progressive rise in Hb F until F cells and F reticulocytes achieved very high levels (FIG. 3). Later, Stamatoyannopoulos and co-workers, who combined pulses of cell cycle–specific drugs with pulses of erythropoietin, also achieved high levels of Hb F production in simians.[10] Five years ago, Platt and co-workers demonstrated a prompt rise in Hb F production in two patients with sickle cell anemia,[11] and a larger series of patients has recently been presented by the groups at Johns Hopkins University and at the National Heart, Lung and Blood Institute

(NHLBI).[12,13] It is now very clear that—for reasons still poorly understood—in patients with accelerated erythropoiesis interruption of the S phase of erythropoiesis by drugs such as hydroxyurea stimulates γ-chain accumulation in erythroid cells. It is doubtful that hydroxyurea-induced γ chain production will influence the transfusion requirement of patients with severe β-thalassemia, but that remains to be seen.

It remains possible, however, that certain relatively non-toxic cytokines may be found that will influence γ-chain production in some patients. That cytokines may influence gene expression is well established. An excellent example has been developed by Ezekowitz and co-workers, who have shown that a pulse dose of γ-interferon administered *in vivo* can lead to a rapid rise in superoxide production by the defective granulocytes in chronic granulomatous disease.[14] The increase continues for 10 or 15 days after the administration of γ-interferon and may persist for another 20 days before a decline. Even in severe cases, a rise in granulocyte superoxide production may be seen for up to 20 days. This long time-course strongly suggests that the γ-interferon alters the program of the oxidase gene in these defective cells at the progenitor level. Indeed, Ezekowitz and Sieff and co-workers have demonstrated that that is exactly the case.[15] Progenitor-derived colonies of granulocytes and macrophages cultured from the blood of patients with chronic granulomatous disease demonstrate increased nitroblue tetrazolium (NBT) reduction for weeks after γ-interferon administration. This is an extraordinary example of successful manipulation of gene expression at the progenitor level, and it provides hope that similar manipulation of globin gene expression might one day be possible.

In conclusion, there is reason to be optimistic about pharmacologic manipulation of γ-globin gene expression. Far more is known about such opportunities than could have been hoped for at the Fifth Cooley's Anemia Symposium held five years ago. The next five years should be challenging indeed.

ACKNOWLEDGMENTS

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Pharmacologic Manipulation of Fetal Hemoglobin

Update on Clinical Trials with Hydroxyurea

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INTRODUCTION

It has long been appreciated that increases in fetal hemoglobin (Hb F) levels in patients with hemoglobinopathies such as sickle cell disease and β-thalassemia major may lead to amelioration of these diseases. In sickle cell disease, increases in Hb F decrease the concentration of hemoglobin S within the red cell, thereby decreasing the tendency for hemoglobin S to polymerize. In β-thalassemia, increases in Hb F production offset the imbalance between α and β chains. With the observation of DeSimone, Heller, and co-workers that anemic animals could increase their Hb F levels after administration of 5-azacytidine,[1] it became clear that the potential for elevating Hb F levels with chemotherapeutic agents was at hand.

Since 1982, a variety of agents have been shown to increase Hb F in animals, in humans, or in erythroid culture. These agents can be divided into two broad categories. First, there are cytotoxic agents, including 5-azacytidine,[1-5] hydroxyurea,[6-9] cytosine arabinoside,[8,10] methotrexate,[11] and myleran.[12] Although the mechanisms by which cytotoxic agents increase Hb F remain unclear, two hypotheses predominate. The first suggests that these agents are toxic, if not lethal, to the more mature erythroid precursors. This toxicity leads to recruitment of earlier progenitors with greater potential for making Hb F.[13-15] The second hypothesis suggests that interruption of the cell cycle might have direct effects on differential gene expression, thereby leading to increased Hb F production.[16-18] The second group of agents which increases Hb F is the humoral growth factors.[19-22] These factors have clearly been shown by Stamatoyannopoulos and colleagues to increase recruitment of earlier erythroid precursors, thereby leading to increased Hb F production. Sodium butyrate and its analogues also increase Hb F production.[23,24] These compounds do not fall into either of the previous categories but may have direct action on chromatin leading to altered gene expression.

CLINICAL TRIALS

5-Azacytidine

Following initial observations that Hb F levels could be raised with 5-azacytidine, our laboratory along with collaborators at several institutions treated 17 patients who had sickle cell disease with this drug.[3-5] The National Institutes of Health (NIH) group under the direction of Arthur Nienhuis treated four patients who had

thalassemia with 5-azacytidine and observed in two of the four patients an increase in Hb F levels.[2,25] Because of the concern about the potential carcinogenicity of 5-azacytidine and because of the advent of potentially safer agents which increase Hb F (hydroxyurea, cytosine arabinoside), clinical protocols utilizing 5-azacytidine were discontinued. Prior to discontinuation of these trials, it was clear that most if not all patients with sickle cell disease responded with increased Hb F production on 5-azacytidine therapy. However, the response of thalassemia major patients to 5-azacytidine was variable, and no patient's response was sufficient to decrease the transfusion requirements.[25]

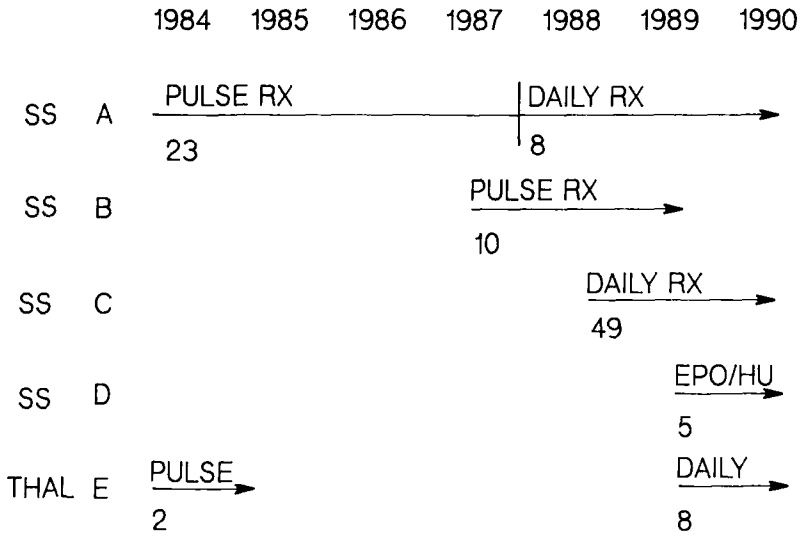

FIGURE 1. Summary of hydroxyurea clinical protocols for sickle cell (SS) patients followed at Johns Hopkins University Medical School (JHU) and collaborating institutions. (**Protocol A**)[9,18] G. Dover and S. Charache, JHU; O. Platt, Harvard Medical School; R. Nagel, Albert Einstein College of Medicine; S. Ballas, Thomas Jefferson University School of Medicine; P. Milner, Medical College of Georgia. (**Protocol B**)[28] G. Dover, JHU; G. Rodgers, C. Noguchi, A. Schechter and A. Nienhuis, NIH. (**Protocol C**)[27] G. Dover and S. Charache, JHU; O. Platt, Harvard Medical School; M. Koshi, University of Illinois School of Medicine; E. Orringer, University of North Carolina School of Medicine; W. Rosse, Duke University School of Medicine; P. Milner, Medical College of Georgia. (**Protocol D**) G. Dover and S. Charache, JHU; M. Goldberg and F. Bunn, Harvard Medical School. (**Protocol E**)[25] G. Dover, JHU; A. Nienhuis, T. Ley and K. McDonagh, NIH. THAL, thalassemia; EPO, erythropoietin; HU, hydroxyurea.

Hydroxyurea

Letvin and colleagues[6] first observed that hydroxyurea increased Hb F production in animals, and Platt[7] and colleagues demonstrated that hydroxyurea could increase Hb F in sickle cell patients. To date, over seventy patients with sickle cell disease have been treated on various protocols with hydroxyurea at Johns Hopkins University School of Medicine and collaborating institutions (see FIG. 1). Initial experience with hydroxyurea suggested that sickle cell patients had a variable

TABLE 1. Summary of Hb F Parameters in Two Sickle Cell Patients Treated with Hydroxyurea

Dose[a]	Days	% Hb F	% F Reticulocytes	% F Cells	Hb F/F Cell	MCH (Pg)[b]
Patient A						
Pre	—	2	4	10	5	34
Pulse	1254	12 ± 3	23 ± 8	50 ± 11	10	41
Daily	980	21 ± 5	33 ± 9	76 ± 9	13	46
Patient C						
Pre	—	3	14	17	6	34
Pulse	1276	18 ± 2	30 ± 8	67 ± 7	12	43
Daily	916	21 ± 5	27 ± 8	77 ± 15	12	43

[a] Pre, prior to treatment; Pulse, weekly pulse therapy; Daily, daily treatment.
[b] MCH, mean corpuscular hemoglobin.

response to hydroxyurea in regards to their elevation of Hb F. Alter and Gilbert[26] observed in hydroxyurea-treated chronic myelogenous leukemia (CML) patients that Hb F levels were higher in patients treated with daily therapy compared to those for patients receiving weekly pulse therapy. In 1987, all sickle cell disease patients at Johns Hopkins were transferred to daily treatment regimens.

Two patients have been treated continuously with hydroxyurea at Johns Hopkins for over 2,000 days. A summary of Hb F parameters monitored in those patients is given in TABLE 1. In Patient A, Hb F dramatically increased on pulse doses of hydroxyurea. However, the Hb F levels nearly doubled when the patient was placed on daily therapy as compared to pulse therapy. Patient C had a dramatic response to pulse therapy with hydroxyurea which did not increase significantly when this patient was placed on daily therapy. Over a four year period of time, 5 out of 23 patients treated with pulse therapy on hydroxyurea have increased their Hb F levels to greater than 20%. In comparison, 5 out of 8 sickle cell patients have increased their Hb F levels to >20% when treated with low-dose daily therapy. By 1988 it had become clear that hydroxyurea in low-dose daily regimens could significantly increase Hb F levels in most patients with sickle cell disease. At that time, a Phase I–II prospective clinical trial was initiated to determine the dose-versus-Hb F response for a larger number of patients on daily therapy. The clinical trial was begun in October 1988 and will be completed in June 1990 (see Protocol C, FIG. 1). Preliminary data from this clinical trial have been presented,[27] and it is clear that hydroxyurea will increase Hb F levels significantly in patients treated for more than one year. A second group of patients have been treated in collaboration with Drs. Rodgers, Noguchi, Schechter, and Nienhuis at the NIH (Protocol B, FIG. 1). In 10 patients, 7 significantly increased their Hb F levels on pulse therapy. These were treated for 90 days as inpatients, and Hb F levels in responders increased by greater than fourfold over a 90-day period.[28]

On the basis of these trials, it has become clear that hydroxyurea will raise Hb F levels in some patients with sickle cell disease to a level which should ameliorate the severity of their disease. However, until a control blinded clinical trial is performed, it will be unclear whether this drug will be therapeutic for a majority of patients with sickle cell disease. In all studies, maximal Hb F levels have been attained with minimal, rapidly reversible myelotoxicity and no increase in infections.

Hydroxyurea Therapy with Thalassemia

The early trials with hydroxyurea in patients with thalassemia have been disappointing. As reported by Nienhuis and colleagues at the previous Cooley's Anemia Symposium,[25] the first two patients treated with pulse therapy with hydroxyurea showed very little response in increasing their Hb F levels and no decrease in their transfusion requirements. Because of the observation in sickle cell patients that daily hydroxyurea therapy increases Hb F levels to a greater extent and with less toxicity than does pulse therapy, several thalassemia patients are presently under therapy at the NIH with daily hydroxyurea treatment. One patient followed at the University of North Carolina by Dr. Orringer in a protocol developed at the NIH by Nienhuis has shown increases in the % F cells, % F reticulocytes, and % Hb F (see FIG. 2). Two features distinguish this patient's response from that of earlier patients tried on hydroxyurea. First, the thalassemia patient is being treated with low-dose daily therapy rather than with pulse therapy. Second, this is a patient with thalassemia intermedia who has baseline hemoglobin values between 4 and 6 g/dl. This may suggest that daily therapy with low-dose hydroxyurea in patients with some β-globin production might lead to increases in γ gene expression sufficient to offset the α:β globin chain imbalance. Further studies using daily therapy in patients with severe transfusion-dependent β-thalassemia and in patients with thalassemia intermedia are under way.

Erythropoietin and Hydroxyurea

Al-Khatti and colleagues pointed out that high-dose erythropoietin treatment stimulated Hb F production in animals and in patients with sickle cell disease.[20,22,29]

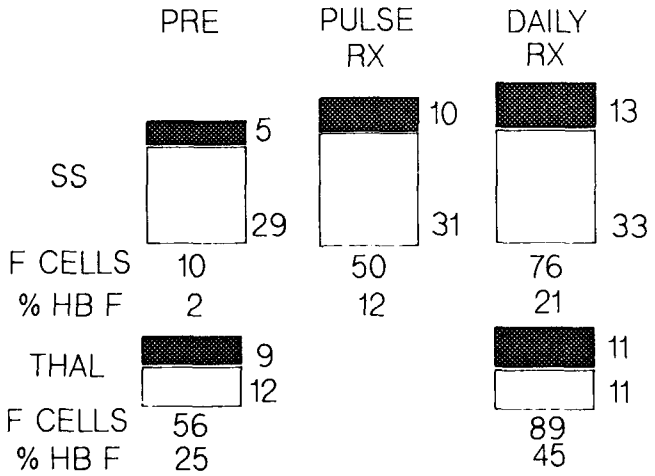

FIGURE 2. Comparison of hydroxyurea-induced changes in mean corpuscular hemoglobin of F cells, in % F cells, and in % Hb F in a sickle cell (SS) and a thalassemia intermedia (THAL) patient. *Bar graphs,* mean picograms of hemoglobin in F cells. *Cross-hatched areas,* picograms of Hb F per F cell. *Numbers to the right* of each bar graph refer to picograms of Hb F, Hb S (SS patient), or Hb A (THAL patient) in the F cells. PRE, prior to treatment; PULSE RX, weekly pulse therapy; DAILY RX, daily treatment.

On the basis of these observations, Drs. Bunn and Goldberg have collaborated with Dr. Sam Charache and myself in treating sickle cell patients with erythropoietin alone, followed by hydroxyurea alone, and then followed by a combination of erythropoietin and hydroxyurea. Seven patients have entered the protocol; only three have so far completed it (Protocol D, FIG. 1). These patients show significant increases in Hb F production in response to hydroxyurea with little or no response to erythropoietin. Combinations of hydroxyurea and erythropoietin did not appear to increase Hb F levels substantially over those obtained with hydroxyurea alone. It remains to be seen whether erythropoietin alone or in combination with other agents such as hydroxyurea might increase Hb F production in patients with thalassemia.

CONCLUSION

A wide variety of pharmacologic agents have been shown to increase Hb F levels either in animals, in humans, or in erythroid cultures. These agents offer some hope that a combination of agents or a new single agent might someday be found that will substantially increase Hb F both in patients with sickle cell disease and in those with thalassemia. That hope seems closer to a reality in patients with sickle cell disease. Indeed, clinical trials have progressed far enough to consider doing definitive control clinical trials of hydroxyurea in patients with sickle cell disease. The progress has not been as great for patients with thalassemia. Differences in response probably relate to the pathophysiology of these two disorders. Hb F levels probably need to be increased to between 15% and 25% of total non–α-globin to have a significant clinical effect in patients with sickle cell disease. However, a comparable increase of Hb F in patients with thalassemia and no β-globin production is unlikely to be sufficient to offset the deficiency in β chains or to result in more effective erythropoiesis. However, hydroxyurea may prove to be effective in patients with thalassemia intermedia who have a significant, albeit decreased, level of β-globin synthesis.

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Regulation of γ-Globin Expression in Hereditary Persistence of Fetal Hemoglobin

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INTRODUCTION

In humans, fetal (γ) globin chains are almost completely replaced by adult (β and δ) globin chains at birth; the molecular mechanisms underlying the switch to adult globin synthesis are unknown. Understanding these mechanisms might be of considerable practical value, as it could lead to the development of methods for maintaining elevated levels of functional fetal hemoglobin (Hb F, $\alpha_2\gamma_2$) in patients suffering from inherited hemoglobinopathies, like β-thalassemia and sickle cell disease. A natural model for the study of mechanisms causing high Hb F production after birth is represented by a clinically mild and genetically heterogenous condition known as hereditary persistence of fetal hemoglobin (HPFH). A subclass of this condition is characterized by persistent overexpression of only one of the two non-allelic γ-globin genes ($^{G}\gamma$ and $^{A}\gamma$) and by point mutations in the promoter of the overexpressed gene[1,2]; strong genetic evidence indicates that at least some of the identified mutations must be responsible for the HPFH phenotype.[1,2] Mechanisms underlying the overexpression of some of the mutated HPFH promoters have recently been suggested; a T→C mutation at position −175 of the γ-globin gene results in better *in vitro* binding to the mutated DNA region[3,4] by an erythroid-specific factor[5] (known as NFE-1[6] or GF-1[4]) and in slightly increased (4–5-fold) activity of the mutated promoter in transfection experiments.[4,7] Similarly, the −198 T→C HPFH mutation results in increased binding of the ubiquitous factor Sp1 (and of an additional unidentified nuclear protein) to the mutated region and in increased activity (4–5-fold) of the promoter in transfection experiments.[8] However, better binding of nuclear factors to mutated promoters may not always be the mechanism underlying HPFH; at least two HPFH mutations (−117 G→A and a deletion of 13 nucleotides, −117 to −105) result in a complex pattern of changes in *in vitro* binding of nuclear proteins characterized by increased binding of certain factors as well as decreased binding of other factors,[3,6,9,10] suggesting that loss of negative effects may be important in causing HPFH. One critical issue in the various models proposed is the functional role of the binding of the erythroid factor NFE-1 to its binding site, adjacent to certain of the HPFH mutations (the −117 and the 13-nucleotide deletion). In this work, we have destroyed the NFE-1 binding site in this region, and we show that this mutation results in a substantial decrease of the activity of the γ-globin promoter.

The implications of this observation for the molecular basis of HPFH and the possible role of an additional factor, NFE-3,[10] are discussed.

METHODS

Plasmids for chloramphenicol acetyltransferase (CAT) assays contain a fragment of the human γ-globin promoter (from −299 to +35) joined by linkers to the *Hin*d III site of the plasmid pSVo-CAT[11]; γ-globin promoter mutants have been described previously. The new mutants (−104 G→A) were obtained by using (in conjunction with a downstream oligonucleotide) an oligonucleotide carrying this mutation to prime the *in vitro* amplification (by polymerase chain reaction, PCR) of the γ-globin promoter fragment located between position −146 and the *Hin*d III linker joined the 3′ end of the promoter to the pSVo plasmid. Following enzyme digestion, a *Nco* I–*Hin*d III fragment of the amplified DNA was used to replace the corresponding sequence in the previously constructed mutants. Transfection of K562 cells by electroporation and CAT assays were carried out as previously described,[7] using the [^{14}C]chloramphenicol butyrylation test.[12]

RESULTS

At least four different nuclear proteins (NFE-1, NFE-3, CP-1, CDP) are known to be able to bind *in vitro* to sequences (from −122 to −98) adjacent to the −117 and 13-nucleotide-deletion HPFH mutations[2,3,6,9,10] (FIG. 1). NFE-1, the best-characterized erythroid-specific factor, binds to a "core" GACAAGG motif (−104 to −98) and also contacts the upstream guanine at position −117; the substitution for this guanine of adenine in the −117 HPFH promoter results in significantly decreased NFE-1 binding.[6] The importance of this effect for the function of the γ-globin promoter cannot by easily assessed, as the −117 HPFH mutation also affects the binding of the other three proteins.[6,10] We therefore mutated the "core" NFE-1 recognition motif, changing the guanine at position −104 to an adenine; this mutation destroyes the NFE-1 binding site, but has little or no effect on CDP and CP-1 binding.[6] The −104 G→A mutation was introduced into the normal γ-globin promoter and into previously constructed promoters containing upstream mutations.[7] The doubly mutant promoters (FIG. 2) are denoted dNFE1−/pNFE1−, OTF1−/pNFE1−, −175 HPFH/pNFE1−, and the triply mutant, dNFE1−, HPFH/pNFE1−. In the dNFE1−/pNFE1− promoter, the distal (−192 to −168) and the proximal (−104 to −98) NFE-1 binding sites are inactivated, while in the OTF1−/pNFE1− promoter, the binding site for the ubiquitous octamer-binding factor

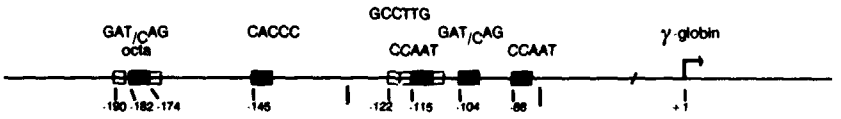

FIGURE 1. Schematic representation of nuclear protein binding sites in the human γ-globin promoter. GAT/$_C$AG, NFE-1 binding site; octa, octamer (OTF-1 binding site); CACCC, CACCC-box protein and Sp1 binding site; GCCTTG (*open boxes overlapping with CCAAT*), NFE-3 binding site; CCAAT, CP-1 binding site. The CDP binding site extends over the whole duplicated CCAAT-box region.[6]

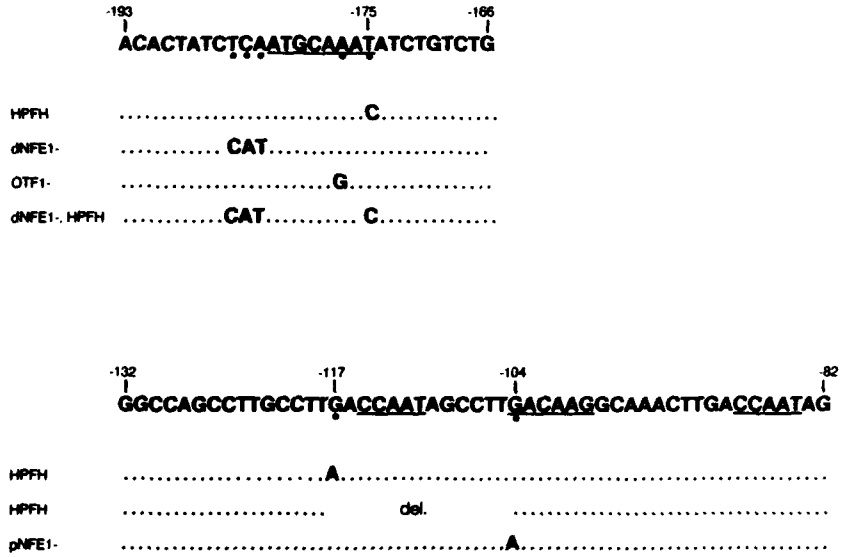

FIGURE 2. Nucleotide sequences of the distal and proximal NFE-1 binding site regions of the promoter, and of HPFH and *in vitro* constructed mutations. dNFE1− and pNFE1−, mutations in the distal and proximal NFE-1 sites, respectively.

(from −182 to −175) and the proximal NFE-1 site are destroyed.[7] The −175 HPFH/pNFE1− doubly mutant promoter is characterized by a better distal NFE-1 binding site[3,4] and a very weak[3,9] OTF-1 binding site (both effects directly resulting from the −175 T→C HPFH mutation) in conjunction with the inactivated proximal NFE-1 binding site. Finally, the triple mutant dNFE1−,HPFH/pNFE1− carries the distal mutation inactivating the NFE-1 site of the −175 HPFH, together with the proximal mutation; it is known that the introduction of the distal NFE1− mutation into the −175 HPFH promoter decreases its functional activity to a level similar to that of a normal γ-globin promoter.[7]

The mutant promoters described above, placed upstream to a CAT reporter gene in the plasmid pSVo,[11] were transfected into human erythroleukemic cells K562. The disruption of the proximal (−104 to −98) NFE-1 binding site abolishes the increased activity of the −175 HPFH plasmid and greatly decreases the expression of the normal and OTF1− promoters (TABLE 1 and data not shown); the activity of the dNFE1−,HPFH (TABLE 1) and dNFE1− promoters (not shown) is decreased almost to background levels by the further introduction of a mutation in the proximal NFE1 site.

DISCUSSION

NFE-1/GF-1 is an erythroid-specific factor capable of binding *in vitro* not only to the γ-globin promoter[3,5,6] but also to the β-globin promoter and enhancer[13,14] and to some non-globin, but erythroid-specific, promoters.[15–17] A NFE-1 binding site in the erythoid-specific promoter of the porphobilinogen deaminase gene is essential for its activity,[16] and deletion of a DNA fragment of the β-globin promoter including an

NFE-1 binding site also results in substantial loss of activity.[13] The role of the two NFE-1 binding sites in the γ-globin promoter is not well defined. Inactivation[4,7] or deletion[18] of the distal NFE-1 binding site causes little, if any, decrease of the activity of the promoter; this result suggests that, at least in the erythroleukemic cells used in transfection experiments, the distal NFE-1 site does not significantly contribute to the overall activity of the promoter. On the other hand, the −175 HPFH mutation, generating a better NFE-1 site, increases the erythroid-specific activity of the γ-globin promoter. In this case, the distal NFE-1 binding site is clearly essential, as the introduction of a mutation inactivating it in the −175 HPFH promoter abolishes its increased activity.[4,7]

Our observation (TABLE 1) that disruption of the proximal NFE-1 binding site abolishes the increased activity of the −175 HPFH mutant and decreases almost to background level the activity of the normal and OTF1− (and, in particular, the distal NFE1−) promoters shows that the proximal NFE-1 site is the major determinant of

TABLE 1. Effect of Mutant Promoters on CAT Reporter Gene Activity in Transfected K562 Cells

Promoter	CAT Activity[a] (conversion × 10^{-3})		Ratios of Activity[b]
Normal	7.9 9.2	8.6	1
−175 HPFH	41.4 20.6	31	3.6
HPFH/pNFE1−	4.7 12	8.4	1
dNFE1−,HPFH/pNFE1−	1.8 4.3	3.1	0.4
OTFI−/pNFE1−	5.8 3.5	4.7	0.5

[a]Chloramphenicol butyrylation by extracts from transfected cells. Values are given as proportion of [^{14}C]butyryl-chloramphenicol out of total chloramphenicol (0.5 μCi) after subtracting the corresponding values obtained with pSVo. Results of a typical experiment are given; values to right are averages. In this experiment, the pSVo conversion value was 3.4.

[b]Ratios of activity are calculated relative to the normal γ-globin plasmid.

the erythroid-specific expression of the γ-globin gene. This conclusion has important implications for our understanding of HPFH; the −117 HPFH mutation decreases NFE-1 binding,[6] and it was suggested that NFE-1 might behave as a negatively acting factor when bound to this site. Our results do not support this interpretation, and rather suggest that altered binding of other factors may be relevant to the HPFH phenotype. Indeed, the −117 HPFH mutation causes increased *in vitro* binding of CDP and CP-1 and decreased binding of NFE-1 and NFE-3. On the other hand, HPFH due to the deletion of nucleotides −117 to −105 results in essentially normal binding of NFE-1 and decreased or abolished binding of CDP, CP-1 and NFE-3.[10] These results suggest that decreased binding of NFE-3 (the only common effect of the two HPFH mutations) may be important for the HPFH phenotype. Using NFE-3 partially purified by affinity chromatography, we recently observed by dimethyl sulfate (DMS) interference experiments that the guanine at position −117 is important for NFE-3 (data not shown) as well as for NFE-1 binding,[6] and preliminary

evidence suggests that the two factors may not bind simultaneously to the same DNA fragment. Whether NFE-3 can functionally antagonize the erythroid-specific activity of NFE-1 at the proximal site is not known and is presently being investigated.

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A Model for Reactivation of Hemoglobin F Synthesis in Normal Adult Erythropoiesis[a]

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INTRODUCTION: *IN VIVO* AND *IN VITRO* REACTIVATION OF HEMOGLOBIN F SYNTHESIS

In the perinatal period, fetal hemoglobin (Hb F, $\alpha_2\gamma_2$) is subtotally replaced by Hb A ($\alpha_2\beta_2$) and some Hb A_2 ($\alpha_2\delta_2$). Thereafter, Hb F (< 1% of total Hb) is restricted to F cells, which represent < 6% of red blood cells (RBC).[1,2]

In a variety of postnatal conditions (particularly in marrow regeneration and stress erythropoiesis), Hb F synthesis may be reactivated to a level of up to 10–20% relative to γ-globin content.[3,4] A similar reactivation has been observed *in vitro:* in fetal calf serum–supplemented (FCS^+) semisolid cultures treated with erythropoietin, the erythroid burst-forming units (BFU-E) from normal adults generate erythroblast colonies ("bursts") with a marked enhancement of relative γ-chain synthesis (i.e., to 10–20%),[5] as compared to corresponding *in vivo* levels (< 2–3%). Evaluation of globin production in single BFU-E–derived clones showed that all normal adult bursts synthesize a significant amount of γ chains.[6,7] These results, coupled with a similar analysis of single bursts from yolk sac,[8] embryonic or fetal liver,[8,9] and cord blood[10,11] indicate that post-embryonic BFU-E are always bipotent for Hb F and Hb A synthesis. The Hb F potential obviously prevails in fetal life, but it is gradually and almost totally replaced by the program for Hb A (and some Hb A_2) production in the perinatal period.[10,11] However, the potential for significant Hb F synthesis is maintained in all postnatal BFU-E.[6,7]

It is also noteworthy that in the erythroblast differentiation pathway the synthesis of γ chains peaks earlier than does the production of β-globin in fetal,[9] perinatal, and adult[12] life.

The mechanism(s) underlying reactivation of γ-globin synthesis in normal adult bursts grown in FCS^+ cultures have not yet been identified. Three major limitations have hampered an in-depth analysis of this phenomenon:

1. It requires large amounts of pure hemopoietic growth factors, i.e., a variety of colony-stimulating factors (CSF) and interleukins (IL). These have recently been made available by recombinant DNA techniques.[13]

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2. The use for hemopoietic culture of FCS, which contains unknown hemopoietic growth factors and enhances proliferation of accessory cells releasing endogeneous hemopoietins, complicates both the methodology and the interpretation of results. For this reason, intensive efforts have been devoted in our laboratory to optimizing a FCS^- culture system for cloning of human hemopoietic progenitors. The current system allows adequate BFU-E proliferation and differentiation, at least up to the level observed in FCS^+ clonogenic culture, but allows little proliferation of accessory cells interspersed among colonies.[14,15]
3. There coexists in the plated cell population a miniscule pool of progenitors and a large number of accessory cells releasing unknown quantities of only partially identified endogenous growth factors,[16] which mask the effect of exogenous growth factors and hinder both reproducibility and analysis of data. Therefore, intensive efforts have been devoted to the purification of hemopoietic progenitors. Recently, we have developed a method to purify to homogeneity early hemopoietic progenitors (BFU-E, CFU-GM, CFU-GEMM)[c] from normal human adult peripheral blood mononuclear cells (PBMC; results not presented here). This method is based on three negative and one positive selection step: (1) isolation of PBMC on a Ficoll gradient, followed by stringent removal of monocytes; (2) isolation of light-density cells on a Percoll density gradient; (3) immune absorption to magnetic beads coated with a panel of monoclonal antibodies (mAb) against B, T and NK lymphocytes, monocytes, and granulocytes; and (4) positive selection by immunoadsorption with two monoclonal antibodies (mAb) to CD34 antigen.

We report here a series of studies on Hb F reactivation in normal adult erythroid bursts, as evaluated by addition of recombinant growth factors in FCS^- culture of either PBMC or purified peripheral blood BFU-E.

REACTIVATION OF Hb F SYNTHESIS IS SUPPRESSED IN FCS^- CULTURE OF NORMAL ADULT BFU-E

A large series of experiments has been focused on the comparative analysis of Hb F synthesis in normal adult bursts from PBMC grown in FCS^+ versus FCS^- conditions (TABLE 1). In the presence of saturating amounts of erythropoietin, both systems allow optimal proliferation and differentiation of BFU-E. However, the relative γ-chain synthesis in mature erythroblasts is much lower in FCS^- conditions (2–4%) than in FCS^+ cultures (18–20%). A similar difference is observed for Hb F content[14] and percentage of F cells (FIG. 1).

In FCS^- culture, the number of GM colonies is markedly lower than in control FCS^+ dishes: we suggest that this decrease is due to the reduced level of endogenous hemopoietic growth factors (e.g., GM-CSF and/or IL-3) released by accessory cells in the FCS^- system as compared to the larger amount released in FCS^+ cultures. In this regard, GM-CSF and/or IL-3 is produced by a variety of accessory cells, i.e., T lymphocytes,[13] large granular lymphocytes (LGL),[17] and monocytes-macrophages.[18] FCS^+ cultures seeded with a high density of PBMC (3×10^5 cells/ml) contain a large number of accessory cells interspersed among colonies, i.e., T cells and LGL ($> 10^5$ lymphocytes/dish), as well as monocytes-macrophages (> 500 cells/dish). In these

[c]CFU: colony-forming units; GM: granulocytic, monocytic; GEMM: granulocytic, erythroid, megakaryocytic, monocytic.

TABLE 1. Comparison of Pooled Normal Adult Blood Bursts Grown in Either Standard FCS⁺ or Optimized FCS⁻ Culture

Property[a]	Mean Value ± SEM (n = 15)	
	FCS⁺	FCS⁻
BFU-E colonies/plate	89.3 ± 8.6	101.3 ± 7.3
Cells burst ($\times 10^{-3}$)	12.0 ± 2.7	12.5 ± 2.5
Late erythroblasts (%)	86.3 ± 3.9	84.1 ± 4.1
Hb/cell (pg)	27.3 ± 1.4	27.7 ± 1.1
$\gamma/(\gamma + \beta)$ synthesis (%)	20.6 ± 2.2	2.6 ± 0.3
CFU-GM colonies/plate	23.0 ± 2.2	8.1 ± 1.6

[a]Colony scoring and analysis was at day 14 and day 16 for FCS⁺ and FCS⁻ cultures, respectively. Relative γ-globin synthesis was determined by isoelectric focusing analysis.

cultures, therefore, addition of erythropoietin alone allows efficient burst formation and erythroblast maturation, due to release of endogenous GM-CSF and/or IL-3 by accessory cells (indeed, the absolute GM-CSF and IL-3 requirement of adult BFU-E can be demonstrated only in unicellular FCS⁻ culture.[15]). In the FCS⁻ clonogenic system the growth of accessory cells is drastically reduced[14]: this reduction presumably causes a reduced production of endogenous GM-CSF and/or IL-3. Under these conditions and in the presence of erythropoietin, burst formation is still allowed, but the growth of GM colonies is curtailed.

Following this line of reasoning, the hypothesis was considered that in FCS⁻ culture the reduced production of endogenous hemopoietic growth factors may be responsible for the marked decrease of the relative level of γ-chain synthesis, as compared to the level in FCS⁺ culture.

GM-CSF AND IL-3 REACTIVATE Hb F SYNTHESIS IN NORMAL ADULT BURSTS

In order to test this hypothesis, we added GM-CSF, IL-3, or other recombinant hemopoietic growth factors combined with saturating levels of erythropoietin to

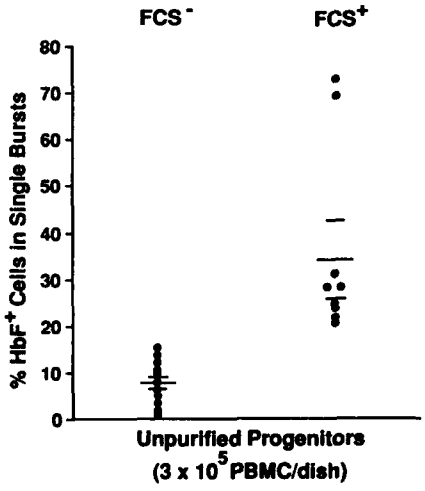

FIGURE 1. Comparison of FCS⁻ and FCS⁺ cultures. Percentage of Hb F–positive (HbF⁺) cells, as evaluated by standard immunofluorescence analysis in single erythroid bursts generated by normal adult PBMC in FCS⁻ or FCS⁺ clonogenic culture (3×10^5 cells/dish) supplemented only with a saturating level of erythropoietin (3.0 IU/ml). For further details see Ref. 14.

normal adult blood BFU-E grown in FCS⁻ culture.[14,19] Addition of GM-CSF (FIG. 2) or IL-3 (FIG. 3) induced a dose-dependent rise of Hb F synthesis in the erythroid bursts, whereas other growth factors (IL-1α or IL-1β, IL-2, G-CSF) had no effect. Increasing amounts of erythropoietin (≥ 10 IU/ml) did not cause a rise of γ-chain synthesis. We cannot exclude, however, that "pharmacological" doses (i.e., > 10 IU/ml) may have some effect, in view of studies indicating that erythropoietin reactivates Hb F synthesis in the baboon.[20,21]

We also investigated the mechanism(s) underlying the reactivation of Hb F synthesis induced by GM-CSF and IL-3. Theoretically, at least four alternative or complementary mechanisms may be envisioned: (1) a defective maturation of erythroblasts in GM-CSF–and/or IL-3–treated dishes, (2) recruitment by these growth factors of a cohort of BFU-E with a higher potential for Hb F synthesis, (3) an indirect effect of GM-CSF and/or IL-3 on erythroid precursors via hemopoietin(s) released by background accessory cells and/or GM colonies, (4) a direct effect of these growth factors on erythroid cells.

The first two mechanisms can seemingly be excluded. In fact, the maturation-block hypothesis is incompatible with control data on the Hb content/cell and percentage of mature erythroblasts (see legends to FIGS. 2 and 3): these indicate an equivalent maturation of erythroblasts in the analyzed FCS⁻ cultures at all GM-CSF or IL-3 dosages, as well as in control FCS⁺ dishes.

The recruitment hypothesis cannot be invoked to explain the results in the experiments with GM-CSF, since the number of bursts/dish was not modified by this growth factor. Furthermore, experiments with IL-3 show that the rise of Hb F synthesis does not significantly correlate with the increase in the number of BFU-E colonies. By process of elimination, then, we suggest that the rise of γ-chain synthesis is mediated by the action of GM-CSF and IL-3 on erythroid precursors, directly and/or indirectly.

The action of GM-CSF or IL-3 on Hb F reactivation was also observed in cultures of partially purified progenitors ($> 20\%$ cloning efficiency, 200 cells/plate), in spite of the absence of background accessory cells and the very low number of GM colonies.[14,19] These preliminary results suggested that the action of GM-CSF and IL-3 is, at least in part, direct. Further experiments have been carried out in clonogenic cultures of purified ($> 90\%$) BFU-E from normal adult PBMC (FIG. 4): the percentage of F cells in single bursts was low after treatment with erythropoietin alone, but it was markedly more elevated upon combined addition of erythropoietin with GM-CSF and IL-3. These experiments conclusively demonstrate that GM-CSF and IL-3 reactivate Hb F synthesis in adult bursts. We cannot, however, exclude the possibility that other cytokines (e.g., IL-6) may potentiate their action on Hb F.

GM-CSF and IL-3 exert their stimulatory action on erythropoiesis at the BFU-E level.[13,22] An effect on later progenitors (CFU-E) or morphologically recognizable precursors has not yet been documented. Thus, we suggest that these growth factors induce *in vitro* the reactivation of Hb F through an action at the level of BFU-E, i.e., via modulation of their Hb F synthesis program.

CYCLING ACTIVITY OF PURIFIED ADULT BFU-E AND REACTIVATION OF Hb F SYNTHESIS

Partially purified BFU-E were cultured for 24 or 48 h in liquid phase supplemented with a large dosage of IL-3 (100 U/ml): this caused a gradual rise in their cycling activity (% [^{3}H]thymidine killing index: 0 h, 13%; 24 h, 23%; 48 h, 29%; 72 h, 42%). Thereafter, the progenitors were seeded at low numbers (500 cells/dish) in FCS⁺ culture, supplemented with a saturating level of erythropoietin and graded

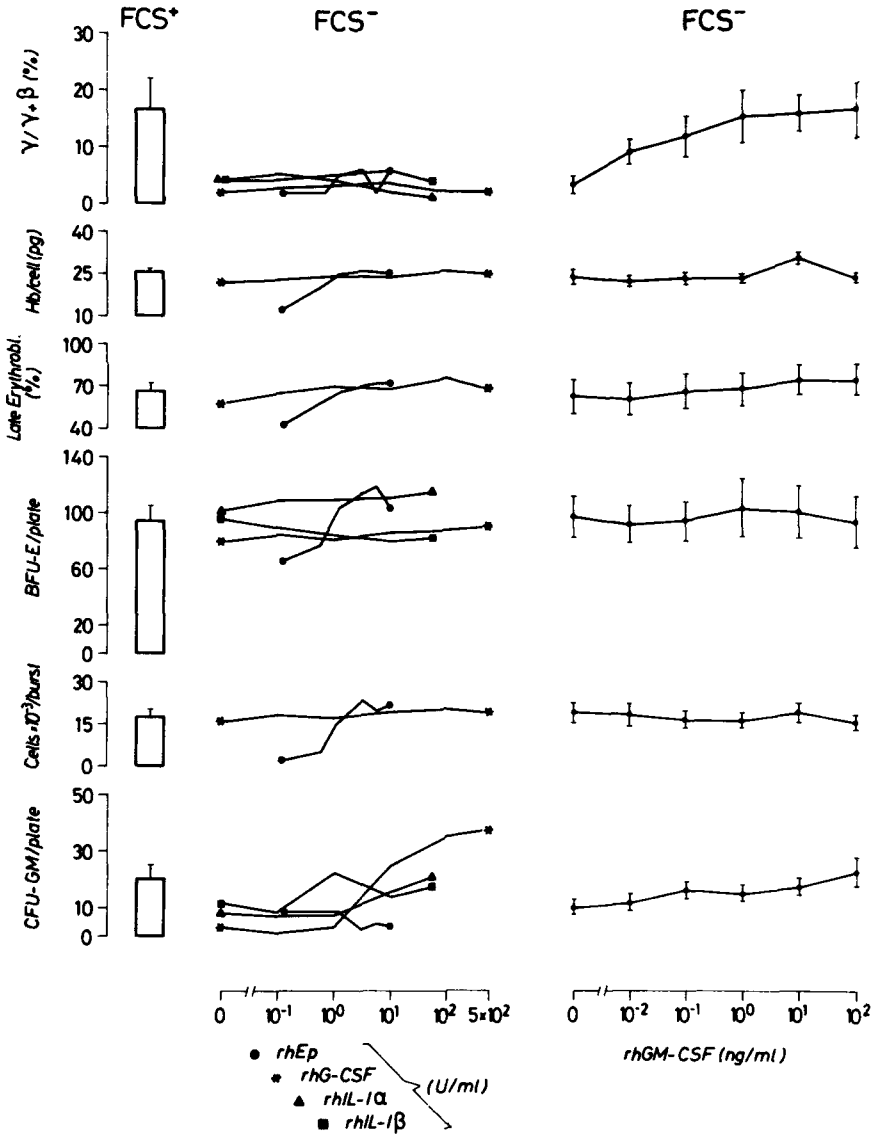

FIGURE 2. Effect of hemopoietic growth factors on FCS⁻ cultures. (**Left panels**) Control FCS⁺ culture treated only with saturating concentration of recombinant human erythropoietin (rhEp; 1.5 IU/dish). (**Center panels**) FCS⁻ culture supplemented with either graded doses of rhEp alone or a saturating level of rhEp combined with graded amounts of recombinant human (rh) cytokines: IL-1α, IL-1β, or G-CSF. (**Right panels**) FCS⁻ culture supplemented with saturating level of rhEp combined with graded doses of recombinant human GM-CSF. Parameters measured under the indicated conditions are (*from top to bottom*): $\gamma/(\gamma + \beta)$ synthesis (% values, by IEF analysis) in pooled erythroid bursts from normal adult PBMC, Hb content/cell, % late erythroblasts, number of erythroid bursts (BFU-E)/dish, number of cells/burst, and number of CFU-GM colonies/dish. Control and GM-CSF data (**left and right panels**) are mean ± SEM values from five separate experiments in five different donors. Representative, single experiments are shown for the other growth factors (**center panel**). In FCS⁺ cultures, "background" cells were comprised of 10^5–10^6 lymphocytes and 0.5–2 × 10^3 macrophages/dish (see text). In FCS⁻ cultures, treated or not treated with GM-CSF, "background" cells were comprised of < 10^2 macrophages/dish and virtually no lymphocytes. (From Gabbianelli *et al.*[14] Reprinted with permission from *Blood.*)

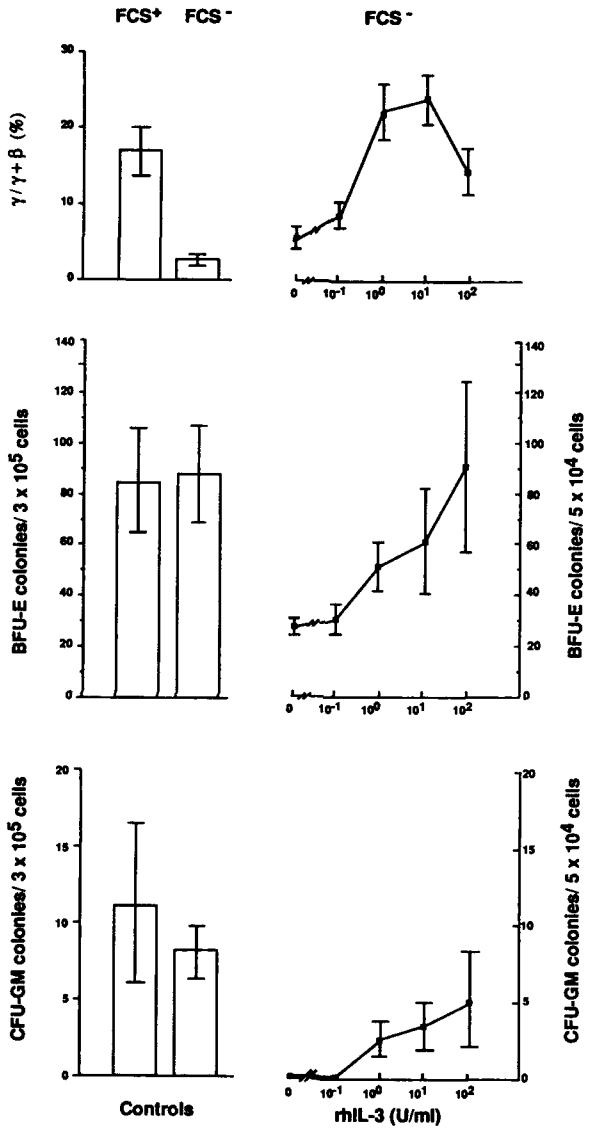

FIGURE 3. Effect of IL-3 on FCS^- cultures. (**Left panels**) Positive (FCS^+) and negative (FCS^-) control cultures (3×10^5 PBMC/plate) treated only with a saturating concentration of erythropoietin (3 IU/ml). (**Right panels**) FCS^- culture of partially purified BFU-E treated with erythropoietin alone or combined with recombinant human IL-3 (rhIL-3; 0.1–100 U/ml). (**Top panels**) IEF analysis of $\gamma/(\gamma + \beta)$ synthesis (% values) in pooled erythroid bursts from partially purified normal adult blood BFU-E (5×10^4 cells/dish) grown in FCS^- culture. (**Middle and bottom panels**) Number of erythroid bursts (BFU-E) and CFU-GM colonies/dish, respectively. Data represent mean ± SEM values from three separate experiments from three different donors. Late erythroblasts in pooled bursts were always >70%. At the end of the culture period, background accessory cells were markedly reduced in all FCS^- cultures (i.e., there were virtually no lymphocytes and $< 10^2$ monocytes-macrophages/dish) as compared to FCS^+ control dishes (10^5–10^6 lymphocytes and 0.5–2×10^3 monocytes-macrophages/dish). (From Gabbianelli *et al.*[19] Reprinted with permission from the *British Journal of Haematology*.)

amounts of GM-CSF. It is apparent that the actively cycling BFU-E primed with IL-3 for 24 or 48 h give rise to erythroid bursts with higher γ-chain synthesis than do the largely quiescent progenitors unprimed with IL-3: the difference is particularly apparent in dishes treated with erythropoietin and not with GM-CSF (FIG. 5).

In further liquid-phase culture experiments, partially purified adult BFU-E treated with elevated amounts of GM-CSF (1–10 ng/ml) similarly showed a gradual although less marked rise of cycling activity and Hb F synthesis potential (data not shown). On the other hand, partially purified BFU-E grown in liquid culture supplemented with low dosages of IL-3 (0.1 U/ml) showed only a borderline rise of the cycling activity and Hb F synthesis potential (data not shown).

In conclusion, these experiments suggest that modulation of adult BFU-E cycling by IL-3 and GM-CSF is directly linked to the Hb F synthesis potential of the progenitors.

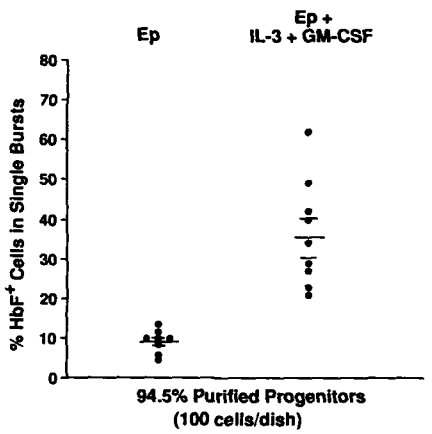

FIGURE 4. Reactivation of Hb F synthesis by IL-3 and GM-CSF treatment. The percentage of Hb F⁺ cells, as evaluated by standard immunofluorescence analysis, in single erythroid bursts generated by 94.5% pure BFU-E, separated from normal adult PBMC and then grown in FCS⁺ clonogenic conditions (100 cells/dish) in the presence of a saturating level of erythropoietin (Ep; 3.0 IU/ml) alone or combined with optimal concentrations of GM-CSF and IL-3 (10 μg and 100 U/ml, respectively).

A MODEL FOR REACTIVATION OF Hb F SYNTHESIS IN ADULT ERYTHROPOIESIS

We propose a unitary hypothesis which is compatible with the above *in vitro* results and may be extended to *in vivo* adult erythropoiesis in both normal and marrow regeneration conditions: this model is based on the concept that the cycling activity of adult BFU-E, modulated by the level of IL-3, GM-CSF and possibly other growth factors, is linked to the potential of the progenitors for Hb F synthesis (FIG. 6).

In Vitro *Studies*

In clonogenic cultures of PBMC, accessory cells are scarce in FCS⁻ conditions but abundant in FCS⁺ medium. Indirect evidence (i.e., the number of GM colonies upon the addition of erythropoietin alone to FCS⁺ or FCS⁻ cultures) suggests that the activity of endogenous GM-CSF, IL-3, and perhaps other growth factors is lower in

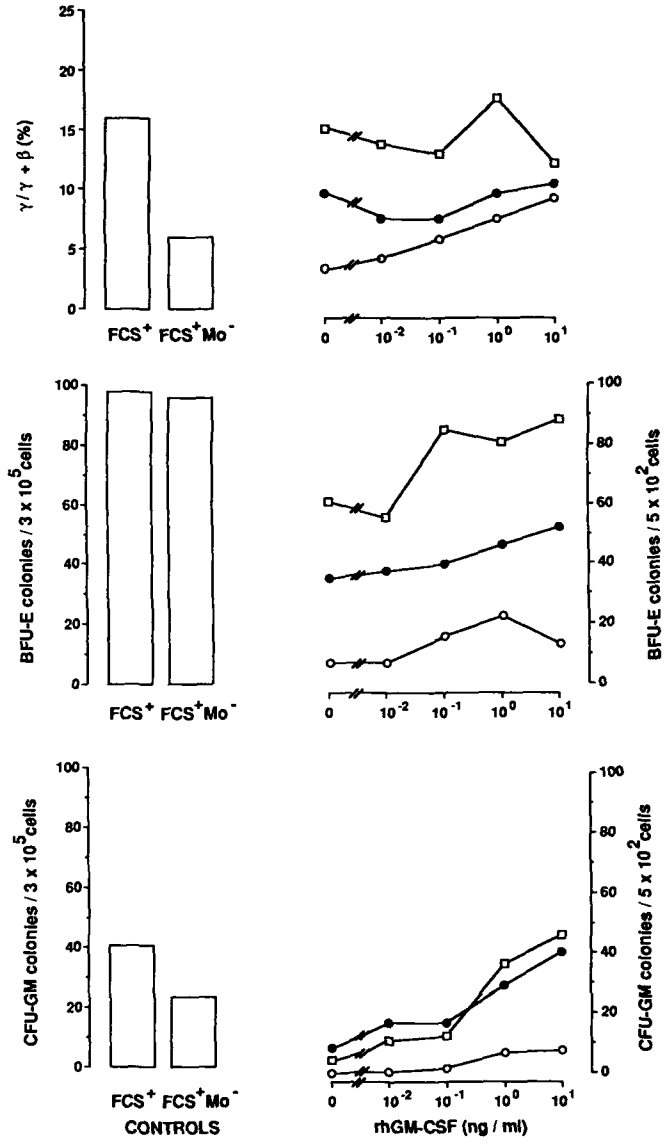

FIGURE 5. Reactivation of Hb F synthesis in actively cycling BFU-E. (**Left panels**) PBMC were grown in FCS⁺ (positive control) or FCS⁺Mo⁻ culture (negative control[14]) in the presence of a saturating erythropoietin level. (**Right panels**) BFU-E partially purified from PBMC were first incubated without IL-3 (○), or with IL-3 (100 U/ml) for 24 h (●) or 48 h (□), in liquid phase culture and then cloned in FCS⁺ conditions in the presence of a saturating level of erythropoietin and graded amounts of GM-CSF. (**Upper panels**) Analysis of γ/(γ + β) synthesis (% values) in pooled erythroid bursts. (**Middle and bottom panels**) The number of BFU-E and GM colonies, respectively. Mo, monocyte.

FCS⁻ culture than in FCS⁺ dishes. The lower level of relative γ-chain synthesis in FCS⁻ versus FCS⁺ culture may be mediated by the corresponding difference in the activity of endogenous hemopoietic growth factors. This postulate has been verified by experiments involving the addition of anti–GM-CSF and/or anti–IL-3 mAb to FCS⁺ cultures of PBMC, which show a dose-dependent decline of the percentage of F cells (results not shown here).

This postulate has been further verified by experiments in FCS⁻ culture involving treatment of PBMC with exogenous GM-CSF or IL-3: both growth factors cause a dose-related rise of relative γ-chain synthesis up to the level observed in FCS⁺ dishes. Elevated dosages of these two cytokines also gradually induce a marked rise of the cycling activity of normally resting BFU-E. It may be suggested, therefore, that large amounts of these cytokines induce an increase of the cycling activity of the progenitors, thus involving a new gene program that leads to an increase in the potential for Hb F synthesis, as expressed in the erythroblastic progeny.

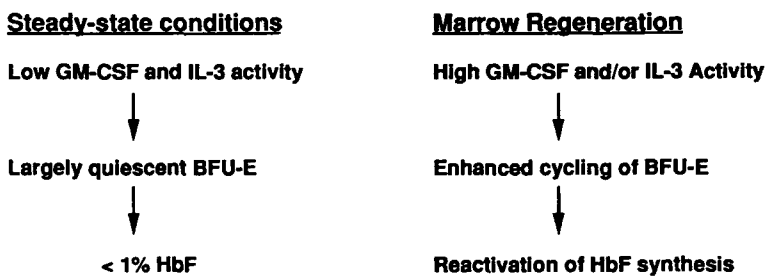

FIGURE 6. A unitary model for reactivation of Hb F synthesis in normal adult erythropoiesis. For further details see text.

A Unitary Model for In Vivo *and* In Vitro *Conditions*

Adult erythropoiesis is associated with the reactivation of Hb F synthesis under conditions of marrow regeneration.[3,4] These conditions are characterized by enhanced cycling of early progenitors, possibly due to increased release of IL-3, GM-CSF, and perhaps other growth factors (e.g., IL-6). Hypothetically, the enhanced activity of these hemopoietic cytokines induces an elevated cycling of BFU-E, which in turn entails a reactivation of the Hb F synthesis program.

The reactivation of Hb F synthesis in marrow regeneration is apparently mimicked by two *in vitro* models: (1) clonogenic culture of unpurified normal adult BFU-E (i.e., PBMC) in FCS⁺ media, which is conceivably associated with a high level of *endogenous* GM-CSF, IL-3 and/or other hemopoietic growth factors; (2) clonogenic culture of unpurified or purified BFU-E in FCS⁻ media supplemented with large amounts of *exogenous* GM-CSF and/or IL-3. Indeed, the level of relative Hb F synthesis observed in marrow regeneration (10–20%) is similar to that observed in these *in vitro* models.

Suppression of Hb F synthesis in normal adult erythropoiesis is associated with a quiescent BFU-E population and, presumably, a low level of GM-CSF, IL-3, and other hemopoietic growth factors (e.g., IL-6). Thus, we suggest that the physiological level of stimulation by these cytokines is low and leads to a largely resting BFU-E

population, which is endowed with a subtotally suppressed Hb F synthesis program. An *in vitro* counterpart of the normal *in vivo* steady-state condition is apparently represented by FCS^- clonogenic culture of unpurified, normal adult BFU-E. This *in vitro* model is characterized by (1) rare growth of accessory cells, (2) an apparently low production of *endogenous* GM-CSF, IL-3 and/or possibly other hemopoietic growth factors, and (3) physiological suppression of Hb F synthesis.

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Function of Transfected Globin Promoters and the Globin Locus Activator in K562 Erythroleukemia Cells[a]

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INTRODUCTION

The $^{G}\gamma$ and $^{A}\gamma$-globin gene promoters are active in fetal erythroblasts and in K562 cells, but their activities decline in adult red blood cells unless a genetic alteration causes one or both of these promoters to be persistently active (hereditary persistence of fetal hemoglobin, or HPFH).[1] The *cis*-acting DNA elements that mediate the transcriptional switch have not yet been defined, since there are no erythroid cell lines that undergo hemoglobin switching *in vitro* and since erythroleukemia cells are difficult to transfect. We undertook these studies to design a highly efficient transient transfection system that would allow us to analyze the function of *cis*-acting elements within the β-like globin gene cluster in an environment where the embryonic and fetal globin genes are constitutively expressed.

We designed a highly efficient transfection system and used vectors that allowed us to map correctly initiated γ- or β-globin mRNA fusion transcripts, with internal controls for transfection efficiency and for quality and content of mRNA. Efficient transfection permitted us to map correctly initiated transcripts from a $^{A}\gamma$-globin promoter fragment fused with the neomycin phosphotransferase gene in a plasmid-based vector (γ-neo) and allowed us to determine that this promoter is enhancer-responsive in K562 cells.[2] The β-globin promoter was inactive in K562 cells, and its function could not be activated by addition of the micro–locus activating region[c] (μLAR) enhancer.[3] These data suggest that this promoter may be repressed in the K562 cellular environment, since the endogenous gene is also inactive. Deletion analysis permitted us to identify two regions in the γ-globin promoter that behave as positive regulatory elements; analysis of HPFH-associated γ-globin promoter mutations revealed no overexpression of these promoters in K562 cells. This system should be useful for further analysis of the *cis*-acting regulatory sequences that contribute to high-level γ-globin gene expression in fetal erythroid cells.

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[c]The locus activating region (LAR) is also known as the dominant control region (DCR).

MATERIALS AND METHODS

The expression vectors used in these studies are shown in FIGURE 1.[2] Basically, they consist of either the γ-globin promoter (extending from −299 to +36 with respect to the transcription initiation site) fused to the neomycin phosphotransferase gene in a pUC9-based vector (γ-neo), or the β-globin promoter extending from −375 to +46 fused to the same gene in the same vector (β-neo). The μLAR constructions

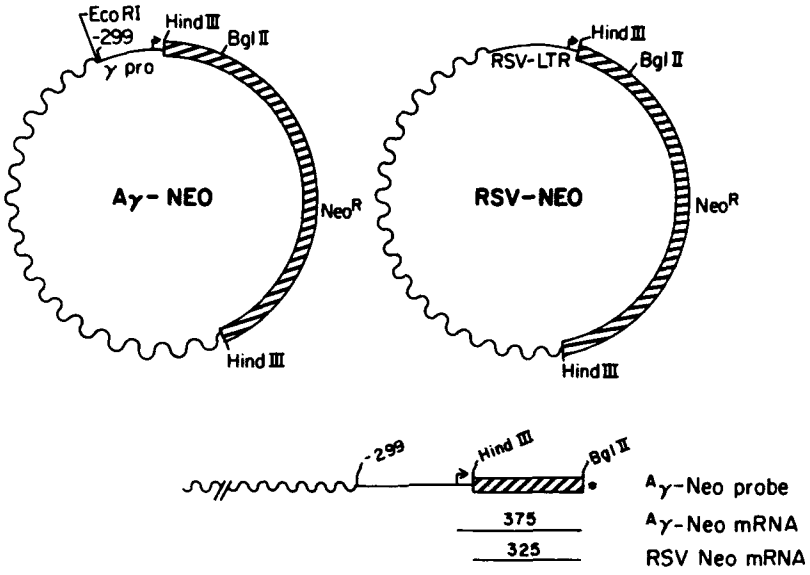

FIGURE 1. Diagrams of the expression vectors used in this study. (**Left upper panel**) Structure of ^Aγ-neo. pUC9 sequences are indicated by a *wavy line,* the γ-globin promoter (γ pro) by a *thin line,* and the neomycin resistance gene (Neo^R) by a *hatched box.* The transcription initiation site of the γ-globin promoter is indicated with an *arrow.* The locations of the *Eco*R I, *Hin*d III, and *Bgl* II sites of the plasmid are shown. (**Right upper panel**) The organization of the RSV-neo plasmid is identical to that described for ^Aγ-neo. Symbols used are as described for ^Aγ-neo, except that *thin line* indicates position of the RSV long terminal repeat (RSV-LTR). (**Lower panel**) The structure of the probe used for S1 nuclease protection assays is shown. The ^Aγ-neo plasmid was cleaved with *Bgl* II, dephosphorylated, and end-labeled as indicated. When this probe is hybridized with correctly initiated ^Aγ-neo mRNA, a probe fragment of 375 nucleotides (nt) is protected from S1 digestion. In contrast, a probe fragment of 325 nt is protected by RSV-neo mRNA, since the RSV-neo promoter diverges from the ^Aγ promoter at the conserved *Hin*d III site. (From Ulrich & Ley.[2] Reprinted with permission from *Blood.*)

were made by releasing a 2.7-kb *Bam*H I fragment containing either the γ-neo or β-neo transcription unit from the parent plasmid and subcloning this fragment into a unique *Bam*H I site located at the 5′ end of the μLAR sequence in a pSP-65–based vector.[3] The γ- or β-neo transcription units were cloned in both orientations within this vector for analysis in these studies. Plasmid DNA used for transfection was banded once in a cesium chloride gradient and then purified using standard

techniques. The quality and concentration of each plasmid was confirmed by agarose gel electrophoresis. Our electroporation protocol was recently described in detail.[2]

RESULTS

Experimental Rationale

As shown in FIGURE 1, in the vector system used for these experiments a wild-type $^{A}\gamma$-globin promoter extending from −299 to +36 was fused to the neomycin phosphotransferase (*neo*) reporter gene. A second plasmid, in which the RSV long terminal repeat was inserted to drive transcription from *neo,* was used as the transfection control (RSV-neo). To detect $^{A}\gamma$-neo and RSV-neo mRNA molecules, we used the probe shown at the bottom of FIGURE 1. A *Bgl* II site is present in both plasmids 325 bp downstream from the *Hin*d III site at the 5′ end of *neo*^{R}. The γ-neo or β-neo plasmids were labeled at this site, total cellular RNA harvested from each transfection point was hybridized with an excess of this probe, and samples were subsequently treated with S1 nuclease. Correctly initiated γ-neo mRNA molecules protect a probe fragment of 375 nucleotides (nt) from S1 degradation, and correctly initiated β-neo mRNA protects a β-neo probe fragment of 385 nt. However, since the γ or β promoters and RSV promoter diverge at the *Hin*d III site, RSV-neo transcripts protect a probe fragment of only 325 nt from S1 digestion. Therefore, transcripts from both cotransfected plasmids can be simultaneously measured. We also added a second probe in the hybridization mixture that detected endogenous γ-globin mRNA. This probe was derived from the normal γ-globin gene and end-labeled at a *Bam*H I site in exon 2. Correctly spliced $^{G}\gamma$- or $^{A}\gamma$-globin mRNA protects a probe fragment of 209 nt from S1 digestion. The presence of this band serves as a control for the quality and content of input RNA. Preliminary experiments were performed using these vectors to optimize for transfection efficiency. We determined that maximal γ-neo and RSV-neo signals were obtained using 80–160 μg/ml (final concentration) salmon sperm carrier DNA in a transfection buffer consisting of serum-free RPMI-1640 medium. Optimal transfection efficiency was obtained with pulses of 250–350 volts at 600–800 μF. In general, most experiments were performed with 250–300 volts at 800 μF of capacitance. Cells were generally harvested 18–24 h after transfection. In separate experiments, we determined that all assays were performed in the linear range of plasmid input.[2]

Deletional Analysis of the γ-Globin Promoter Reveals at Least Two Positive Cis-*Acting Regulatory Elements*

Deletions of the $^{A}\gamma$ promoter were created at positions −299, −247, −199, −160, −130, and −61 with respect to the transcription initiation site, and the 5′ endpoints of all the deletions were sequenced. 10 μg of each plasmid was cotransfected with 2.5 μg of the RSV-neo control plasmid using optimal conditions. RNA was harvested, and S1 protection assays were performed. A representative experiment is shown in FIGURE 2. Deletion to position −160 minimally reduced γ-promoter function in this assay. However, deletion to position −130 decreases the promoter activity to about 20% of that of controls. Further deletion to position −61 essentially abolished γ-promoter activity. These experiments were performed at least five times with essentially identical results; they clearly demonstrate that at least one positive

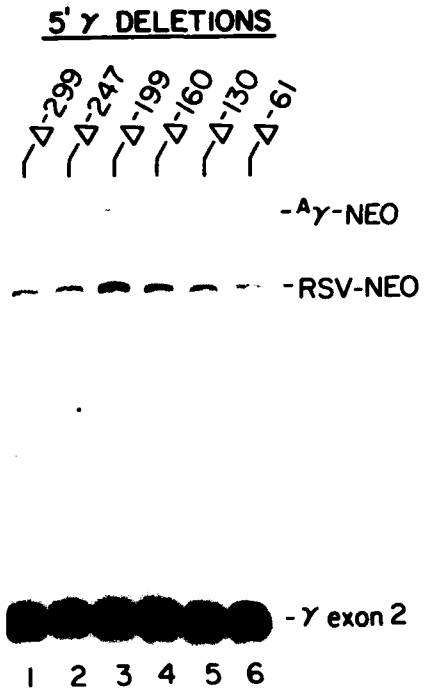

FIGURE 2. Deletion analysis of the $^{A}\gamma$-globin promoter. 10 μg of the $^{A}\gamma$ plasmids (truncated at the indicated positions in the promoter) and 2.5 μg of RSV-neo were cotransfected into K562 cells using optimal conditions, and total cellular RNA was harvested for analysis 24 h after transfection. The sizes of correctly initiated $^{A}\gamma$-neo, RSV-neo, and γ-exon 2 probe fragments are indicated. Note that the $^{A}\gamma$-neo signal changes little with deletion to −160; but it does decline with deletion to −130 and declines even further with deletion to −61. (From Ulrich & Ley.[2] Reprinted with permission from *Blood.*)

regulatory element in the γ-globin promoter lies between −160 and −130 and that at least one more lies between −130 and −61.

Mutations Associated with HPFH Are Not Overexpressed in K562 Cells

A variety of single base changes near the 5′ ends of the $^{G}\gamma$- and $^{A}\gamma$-globin genes have been described in patients with HPFH.[4-8] We wished to determine whether these mutated promoters would be up-regulated in the embryonic-fetal erythroid environment of K562 cells. Mutations at positions −202 (C→G),[4] −196 (C→T),[5,6] and −117 (G→A)[7,8] are all tightly linked with the HPFH phenotype. Another mutation at −199 (T→A) has not been described in patients with HPFH. 20 μg of the mutant $^{A}\gamma$-neo vectors were cotransfected with 2.5 μg of RSV-neo plasmid and harvested for analysis at 24 h (see FIG. 3). Since γ promoter activity can be significantly enhanced with the μLAR or SV40 enhancers,[2] mutations that activate the γ-globin promoter should be capable of being detected by this assay system. However, none of the mutant γ-globin promoters was overexpressed in K562 cells. These results were repeated at least five times with different preparations of mutant $^{A}\gamma$-neo vectors, using DNA inputs of 5–20 μg, with identical results.

Wild-Type γ-neo or −202 C→G γ-neo Is Enhanced by the μLAR But β-neo Is Not

To test the effect of the μLAR on γ-neo expression in K562 cells, we inserted the γ-neo transcription unit into a μLAR plasmid described by Forrester *et al.*[3] The

μLAR was either placed upstream of γ-neo in the opposed orientation (γ-neo μLB) or downstream of γ-neo in the same orientation (γ-neo μLC) (see FIG. 4). Addition of the μLAR to the γ-neo transcription unit dramatically increases the number of correctly initiated γ-neo mRNA molecules; the effect is orientation- and position-independent (FIG. 4, lanes 2–4). As previously shown, the β-neo plasmid is essentially inactive in K562 cells,[2,9-15] as demonstrated in FIGURE 4, lane 5. Addition of the μLAR to the β-neo plasmid does not increase transcription of β-neo. In contrast, the γ-neo μLAR and β-neo μLAR plasmids are both active in stably transformed MEL cells; the β-neo promoter is therefore intact and capable of being expressed (data not shown). The $^{A}\gamma$ promoter containing the −202 C→G substitution is also expressed in transiently transfected K562 cells (FIG. 4, lane 8). When the μLAR is added to the mutant plasmid (lanes 9 and 10), an increased number of correctly initiated γ-neo transcripts is present; the increase is essentially equivalent to that seen with wild-type γ-neo.

DISCUSSION

We have designed a transient transfection system for analysis of *cis*-acting DNA regulatory elements in K562 cells. The deletion analysis of the γ-globin promoter revealed at least two positive regulatory elements. Deletion to position −199 removes a region (−216 to −208) that contains an S1 nuclease–hypersensitive site in supercoiled plasmids, a structure that is altered by HPFH-associated mutations at −202 and −196 (M. J. Ulrich and T. J. Ley, unpublished observations). Deletion to −160, which reduced promoter activity by about 30%, removed a binding site for the consensus octamer sequence and another binding site for the erythroid DNA binding protein GF-1 (also known as eryf-1 or NFE-1).[16,17] Nucleotides from position −160 to

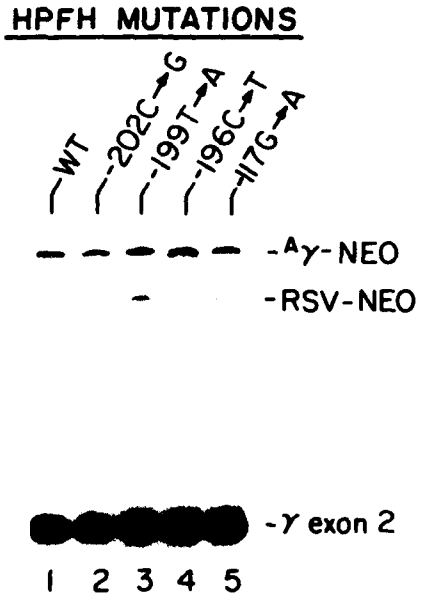

FIGURE 3. Analysis of HPFH promoters. 20 μg samples of $^{A}\gamma$-neo plasmids (all with 5′ ends at −299) containing no mutations (wild-type), or with the substitutions shown, were cotransfected with 2.5 μg of RSV-neo. The positions of correctly initiated $^{A}\gamma$-neo, RSV-neo, and γ-exon 2 probe fragments are indicated. Note that the $^{A}\gamma$-neo signals are essentially identical for all of the mutations tested. The −199 substitution has not been identified in any patient with HPFH. (From Ulrich & Ley.[2] Reprinted with permission from *Blood*.)

−130 contain a consensus for the CACCC motif, first identified as a positive element in the −100 region of the rabbit, murine, and human β-globin genes.[1] This region is located within the DNase I–hypersensitive site just 5′ to the γ genes, so this motif appears to be accessible for the binding of *trans*-regulatory factors.[18] Indeed, Gumuchio, Collins, and colleagues[19] and Catala, Grosveld and colleagues[20] have both detected non-erythroid and erythroid proteins that bind to the CACCC region. All of these studies suggest, but do not yet prove, a specific role for this CACCC box in γ-globin promoter function. Finally, deletion to −61 removes the duplicated CCATT boxes of the γ-globin promoter, again shown to be an important *cis*-acting regulatory element in previous studies of globin promoters.

Analysis of HPFH-associated γ-globin promoter mutations revealed that these promoters are not up-regulated in a fetal erythroid environment. There is no

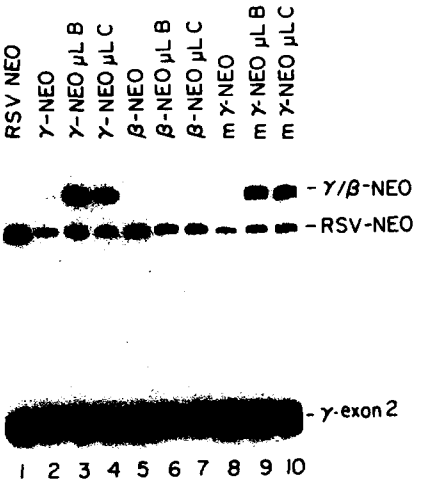

FIGURE 4. Analysis of the effect of the μLAR on γ-neo, β-neo, or γ-neo with the −202 C→G HPFH substitution (m γ-neo). 10 μg of γ-neo, β-neo, or m γ-neo plasmid were cotransfected with 2.5 μg of RSV-neo, or 15 μg of γ- or β-neo with the μLAR (μL) were transfected with 2.5 μg of RSV-neo (increased amounts of the μLAR-containing plasmids were required to maintain molar equivalence of γ-neo). Plasmids designated μLB indicate that the μLAR sequences are upstream of γ-neo or β-neo and opposed in orientation; μLC indicates that the μLAR is downstream and tandem with γ-neo or β-neo. K562 cells were optimally electroporated with these plasmids, and RNA obtained from these cells 24 h later was hybridized with either the γ-neo or the β-neo probe. The position of correctly initiated γ- or β-neo, the position of RSV-neo, and the position of correctly spliced γ-globin exon 2 are indicated. **Lane 1** contains RNA from a transfection with 10 μg of RSV-neo alone. The μLAR significantly increases the abundance of correctly initiated γ-neo or mutant γ-neo mRNA (**lanes 2–4 and 8–10**) but does not activate the β-neo transcription unit (**lanes 5–7**). Separate experiments demonstrated that γ-neo and β-neo functioned essentially equivalently in stably transfected mouse erythroleukemia cells (data not shown).

evidence that individuals with these mutations overexpress their fetal globin genes in fetal erythroid cells or in other tissues. In fact, most studies of transfected γ-globin promoters bearing HPFH mutations have not detected marked overexpression of these promoters in either non-erythroid or erythroid cells.[21–23] One exception is the −175 T→C mutation associated with HPFH, which appears to increase γ-globin promoter activity several fold in chloramphenicol acetyltransferase reporter gene (CAT) assays of transiently transfected K562 cells.[16,24–26] However, these results collectively suggest that most HPFH promoter mutations do not "activate" the γ-globin promoters per se, nor do they activate cryptic enhancers. Alternatively, some of these mutations may act to prevent normal repression of the γ-globin genes in adult erythroid cells.

Several groups, including those of Tuan and colleagues,[27] Forrester, Groudine and colleagues,[3] and Talbot, Greaves, Grosveld and colleagues,[28] have shown that the globin locus activating region behaves as an inducible enhancer when transfected either transiently or stably into erythroid cell lines. Our data clearly demonstrate that the abundance of correctly initiated γ-neo mRNA increases with the addition of the μLAR to the γ-neo plasmid. The wild-type γ-neo plasmid and a plasmid bearing the −202 C→G substitution associated with HPFH behaved in an identical fashion in the K562 cell environment. However, β-neo, which is not expressed in K562 cells, is not activated by the μLAR enhancer, suggesting that the β-globin promoter elements are dominant to the μLAR. Since the μLAR can activate non-erythroid genes like cathepsin G in K562 cells (A. M. Moon and T. J. Ley, unpublished observations), the lack of expression of β-neo suggests that this promoter is actively repressed. Deletion analysis of the β-globin promoter will be required to determine whether a specific repressor element is involved.

Our studies have allowed us to detect positive regulatory elements in the γ-globin gene promoter; and we have determined that HPFH-associated mutations are not overexpressed in the K562 cell environment, regardless of whether the alleles are enhanced with the globin locus activating region. Lack of activation of β-neo by the μLAR suggests that the γ- and β-globin genes are regulated in fundamentally different ways in K562 erythroleukemia cells and underscores the complex interactions between enhancers and promoters that determine the ultimate level of expression of specific globin genes in developing erythroid cells.

SUMMARY

We have examined the importance of *cis*-acting regulatory elements within the human γ-globin gene promoter and the globin locus activating region in K562 cells. ^{A}γ-globin or β-globin promoter fragments were fused with the neomycin phosphotransferase gene in a plasmid-based vector (γ-neo or β-neo) and transiently transfected by electroporation into K562 cells. Correctly initiated γ-neo or β-neo transcripts were detected with an S1 nuclease protection assay that was internally controlled for transfection efficiency and RNA content. We first optimized the conditions for electroporation and then determined that a γ-globin promoter fragment extending from −299 and +36 was active in the assay but that a β-globin promoter extending from −375 to +46 was inactive. Deletion of the γ-globin promoter to −199 did not affect promoter function, but deletion to −160 reduced promoter strength to 70% of that of control. Additional deletion to position −130 reduced promoter strength to 19% of the control value, and to position −61, 8.7% of the control value. Three γ-globin promoters containing mutations associated with hereditary persistence of fetal hemoglobin (HPFH), −202 C→G, −196 C→T and −117 G→A, were not overexpressed in K562 cells, consistent with the hypothesis that these promoters are not overexpressed in fetal erythroblasts, only in adult red cells. When the β-globin locus activating region (LAR) was added to a wild-type or an HPFH γ-neo plasmid, the abundance of correctly initiated γ-neo transcripts increased dramatically. However, β-neo expression could not be activated by the LAR in K562 cells. These studies should allow us to further dissect the interactive roles of globin promoters and enhancers in K562 cells.

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Antenatal Diagnosis of β-Thalassemia in Sardinia[a]

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INTRODUCTION

β-Thalassemias are a group of genetic disorders occurring with a high frequency in the Sardinian population. The incidence of the homozygous state is 1:250 live births, the carrier rate is 1:8; 1 couple out of 60 is composed of two β-thalassemia carriers and thus is at risk of producing a child with the homozygous state referred to as thalassemia major.[1] Thalassemia major is a severe anemia, which, without treatment, leads to death within the first decade. Modern treatment with continuous transfusions and iron chelation by daily subcutaneous infusion with desferrioxamine may permit a long survival. Desferrioxamine treatment, however, is troublesome and associated with low compliance. Bone marrow transplantation from HLA-identical donors may represent an alternative[2,3] but is still associated with a high rate of mortality and/or morbidity.

The high frequency of thalassemia major in the Sardinian population and its severity, combined with the availability of procedures for carrier screening and prenatal diagnosis, led us in 1975 to set up a preventive program based on voluntary carrier screening and prenatal diagnosis[4,5] designed to reduce the incidence of thalassemia major. This paper reviews the characteristics and the results of this ongoing program.

MOLECULAR BASES OF β-THALASSEMIAS IN THE SARDINIAN POPULATION

The first β-globin gene from a Sardinian patient affected by thalassemia major was characterized several years ago by sequence analysis, which revealed the

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presence of a nonsense mutation (CAG→TAG) at the codon corresponding to amino acid 39 (codon 39 nonsense mutation).[6] Later on, studies carried out by oligonucleotide analysis either on non-amplified or amplified DNA led to the definition of the β-thalassemia mutation in 2884 chromosomes from subjects of Sardinian descent (TABLE 1). The most frequent mutation, accounting for 95.7% of the β-thalassemia chromosomes, turned out to be the codon 39 nonsense mutation, followed by the frameshift at codon 6 (codon 6 −1 bp), which accounted for 2.1% of the β-thalassemia defects.[7] Both mutations produce the phenotype of β^0-thalassemia.

Homozygous β^0-thalassemia most commonly produces the phenotype of thalassemia major. In our population, however, in approximately 8%–10% of the cases,[8] homozygous β^0-thalassemia results in a disease of moderate severity referred to as thalassemia intermedia.[9] Studies carried out in the last few years have shown that the homozygous state for the frameshift at codon 6 or the compound heterozygous state for this mutation and the codon 39 nonsense mutation results frequently in a mild phenotype. This may be related to the fact that the frameshift at codon 6 is always contained in haplotype IX. In this haplotype there is a substitution at position −158 5′ to the $^G\gamma$-globin gene that, in conditions of erythropoietic stress, seems to be

TABLE 1. β-Thalassemia Mutations in Sardinians

Type of Mutation[a]	*n*	%
Codon 39 (C→T)	2761	95.7
Frameshift 6 (−1 bp)	60	2.1
IVS-1 nt 110 (G→A)	13	0.5
IVS-2 nt 745 (C→G)	12	0.4
−87 (C→G)	7	0.2
IVS-1 nt 6 (T→C)	3	0.1
IVS-1 nt 1 (G→A)	1	0.03
IVS-2 nt 1 (G→A)	1	0.03
Unknown	26	0.9

[a]A total of 2884 chromosomes were characterized.

associated with high expression of the γ-globin gene.[10,11] The high level of production of the γ-globin chain, by compensating for the absence of β chain production, may explain the mild phenotype. In a limited proportion of the homozygotes for the codon 39 nonsense mutation, the mild phenotype may be explained by the co-inheritance of the deletion of two α-globin structural genes or by point mutations in the α2 globin gene. However, in the remaining cases, which represent the large majority, the molecular basis for the mild phenotype has not yet been elucidated.[9] Therefore, the ability to predict the clinical phenotype of the thalassemia on the basis of the molecular diagnosis is in our population severely limited.

ATYPICAL β-THALASSEMIA HETEROZYGOTES

Heterozygous β-thalassemia commonly results in a hematological phenotype characterized by microcytosis, reduced hemoglobin (Hb) content per cell, high Hb A_2, and unbalanced globin chain synthesis. Two types of heterozygous β-thalassemia that do not fit this definition (atypical β-thalassemia) occur with a relatively high frequency in the Sardinian population. The first, characterized by normal MCV and

TABLE 2. Analysis of Two Methods for Hb A_2 Assay

Subjects	*n*	Hb A_2 (%, mean ± SD) HPLC-Diamat	DE-52 Microchromatography
Normals[a]	452	2.36 ± 0.45	2.34 ± 0.51
β-Thalassemia carriers	369	4.95 ± 0.56	5.02 ± 0.46

[a]Normal subjects include children <6 months old and patients with various hemoglobinopathies (Hb H disease, structural variants).

MCH and by balanced α:β-globin chain synthesis, and defined solely by high Hb A_2, is the result of the co-inheritance of α-thalassemia ($-\alpha/-\alpha,\beta^{thal}\beta^{A}$).[12] Because this type of heterozygous β-thalassemia is easily overlooked in carrier screening by MCH/MCV determination, we carry out Hb A_2 quantitation in each subject. Quantitation of Hb A_2 is obtained automatically by HPLC (TABLE 2), which gives highly reliable results.[13] The second type of atypical heterozygous β-thalassemia is characterized by low MCV/MCH, unbalanced globin chain synthesis, and borderline-to-normal Hb A_2; thus it may be confused with α-thalassemia. This phenotype is the result of the double heterozygous state for δ- and β-thalassemia.[14,15] In the last few years, we have defined the δ-thalassemia mutations in a number of these heterozygotes. The most common mutation turned out to be the G→T substitution at codon 27 (δ^{+27}-thalassemia).[16] In a few cases we detected a deletion of 7201 bp in the ψβ–δ-globin gene region (δ^{0}-thalassemia).[17] The definition of these double δ- and β-thalassemia heterozygotes is, at the present time, accomplished by dot-blot analysis of amplified DNA with oligonucleotide probes specific for δ^{+27}- and deletion δ^{0}-thalassemia (FIG. 1).

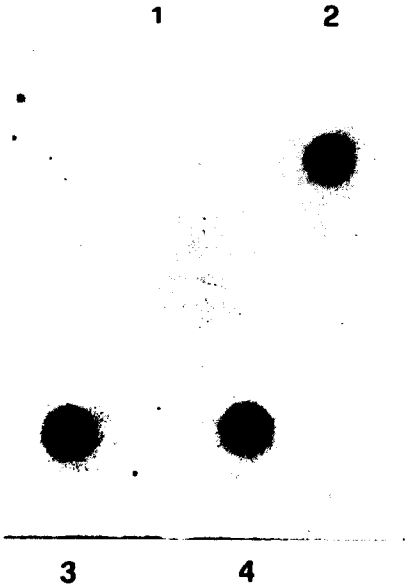

FIGURE 1. Dot-blot analysis of amplified DNA to detect the δ^{+27}-thalassemia (codon 27 C→T) mutation. (**1**) Negative control, (**2**) positive control, (**3,4**) subjects with the δ^{+27} mutation.

CARRIER SCREENING

Carrier screening is carried out voluntarily on couples at child-bearing age with or without ongoing pregnancy. The education of this population at risk is done via mass media (TV and newspapers) and meetings at high schools, industries, and large shops. Special educational meetings are periodically organized to train in this field general practitioners, obstetricians, paramedical professionals, and midwives. By the examination of 167,000 people, we have so far detected 30,500 β-thalassemia carriers (18.3% of those tested) and 1544 couples at risk (TABLE 3). By adding to this figure the number (812) of known couples with children affected by thalassemia major, the total number of couples at risk detected rises to 2356, which represents 87% of the total predicted on the basis of the carrier rate. The high efficiency of our program depends on the wide use of inductive screening, namely extended family examination in all cases in which a person with heterozygous or homozygous β-thalassemia is detected.

In light of the results discussed in the previous section on atypical heterozygotes, for all individuals presenting at our genetic clinic, we carry out a red cell indices analysis with an automatic cell counter (Coulter Counter Model S Plus), automatic Hb A_2 determination by HPLC, and Hb electrophoresis on cellulose acetate. Once

TABLE 3. Overall Results of β-Thalassemia Carrier Screening in Sardinia

Feature	Result
Subjects tested (*n*)	167,000
% of target population tested	24
Carriers detected (*n*)	30,500
At-risk couples detected (*n*)	
Prospective	1,544
Retrospective	812

the couple at risk is identified, we define the mutation in both parents by dot-blot analysis of their amplified leukocyte DNA with ^{32}P- or horseradish peroxidase–labeled oligonucleotide probes complementary either to the codon 39 nonsense mutation or to the frameshift at codon 6. Those cases not defined by this approach are tested with oligonucleotides complementary to rarer mutations.

Counseling is carried out in a non-directive way. In the counseling sessions the natural history of thalassemia major is described. The treatment presently available is presented, and the various options to prevent this disease are discussed. Details on the sampling procedure and its associated risk of fetal loss and on the reliability of the molecular diagnosis are given.

After the introduction of first trimester diagnosis, the large majority of the couples counseled (99%) accepted prenatal diagnosis as a method to monitor for the presence of an affected fetus. In those cases in which a fetus affected by homozygous β-thalassemia was detected, 5 out of 715 (0.7%) women decided to continue the pregnancy because, for ethical reasons, these couples were against the interruption of the pregnancy.

TABLE 4. Prenatal Diagnosis of β-Thalassemia by Fetal Blood Analysis or by DNA Analysis of Non-amplified DNA

Feature	DNA Analysis[a]		Fetal Blood[b]
	Chorionic Villi	Amniocytes	
Pregnancies monitored	953	203	1,130
Homozygous fetuses	261	56	282
Failures	1	6 (3%)	10 (0.9%)
Misdiagnoses	0	0	2 (0.2%)
Fetal losses	26 (2.7%)	4 (2%)	68 (6%)

[a]Diagnosis by hybridization of electrophoretically separated DNA fragments with oligonucleotide probes.
[b]Diagnosis by globin chain synthesis analysis of fetal blood.

PRENATAL DIAGNOSIS

From 1977 to 1983, prenatal diagnosis was carried out by globin chain synthesis analysis of fetal blood, and from 1983 to 1988, by oligonucleotide hybridization on electrophoretically separated DNA fragments (TABLE 4).[18] From 1989, we have been using dot-blot analysis on amplified DNA with oligonucleotide probes complementary either to the codon 39 nonsense mutation or the frameshift at codon 6. Fetal DNA is obtained from either amniocytes or chorionic villi. Chorionic villi are sampled either transvaginally or transabdominally; in our hands, the latter method has proved to be a very safe procedure, with a fetal loss of 2%.

FIGURE 2 illustrates the use of dot-blot analysis to detect the compound heterozygous state for these mutations, and TABLE 5 summarizes the overall results. Chorionic villi DNA analysis with either amplified or non-amplified DNA has proved to be a very reliable procedure. No misdiagnosis has, in fact, so far occurred. Because of

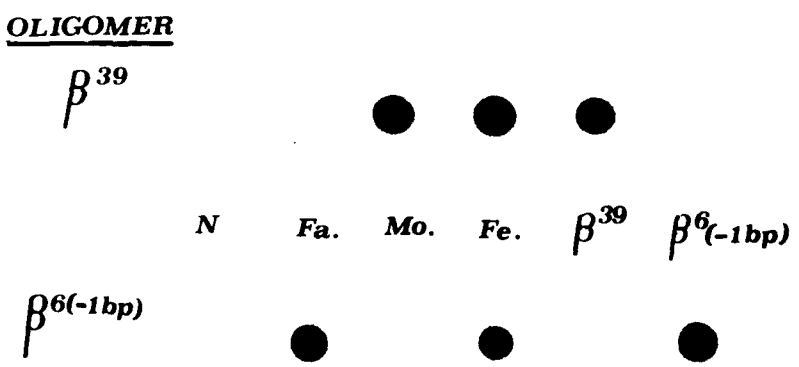

FIGURE 2. Dot-blot analysis of amplified DNA using allele-specific oligonucleotide (ASO) probes complementary to the $\beta^0 39$ and $\beta^0 6$ (−1 bp) mutations. (N), normal DNA; (β^{39}) DNA from a homozygote for the codon 39 nonsense mutation; [β^6(−1 bp)] DNA from a homozygote for the frameshift at codon 6 (−1 bp); (Fa., Mo.) father and mother heterozygous for β6 and β39 mutation, respectively; (Fe) fetus, double heterozygote for these mutations.

the high efficiency of the polymerase chain reaction used to amplify the DNA, concerns have been raised about the possibility of co-amplification of maternal sequences in such a way as to cause misdiagnosis. The most worrisome occurrence, of course, is co-amplification of a maternal sequence in the case of a homozygous fetus, which may thus mistakenly be categorized as a heterozygote. We have carefully analyzed this possibility by carrying out prenatal diagnosis in duplicate on either non-amplified or amplified DNA and by splitting the chorionic villi to be amplified into two samples, which are amplified and analyzed separately.

In the analysis of 425 cases, we have so far detected 4 in which one amplified sample gave a diagnosis of heterozygous β-thalassemia while the other one gave a diagnosis of normal (2 cases) or affected (2 cases). The fetuses were indeed found to be normal or affected, respectively, on the basis of the analysis of non-amplified DNA. These findings clearly indicate that the co-amplification of maternal sequences from maternal leukocytes or decidua may result in misdiagnosis. To avoid this pitfall, it could be useful to amplify simultaneously a highly polymorphic VNTR (variable number of tandem repeats) segment,[19] which may show the contribution of two maternal chromosomes and in this way indicate the presence of maternal contamination.

TABLE 5. Prenatal Diagnosis of β-Thalassemia by Analysis of Amplified DNA

Feature	*n*
Pregnancies monitored	425
Homozygous fetuses	116 (27.3%)
Failures	0
Misdiagnoses[a]	0
Fetal losses	4 (0.9%)

[a]In four cases duplicate amplified samples gave discordant results. Diagnosis was obtained by the analysis of non-amplified DNA.

CONTROL OF β-THALASSEMIA

Carrier screening and prenatal diagnosis have resulted in a rapid decline of the incidence of thalassemia major in the Sardinian population. At the present time, thalassemia major has an incidence of 1:1000 live births, with an effective prevention of 90% of the cases predicted on the basis of the carrier rate (FIG. 3).

The reasons for the residual cases of homozygous β-thalassemia in the Sardinian population are reported in FIGURE 4, which shows that the parents' ignorance about thalassemia accounted for the large majority of the cases (67%), followed by false paternity (13%), and the decision not to interrupt the pregnancy following identification of an affected fetus by prenatal testing (20%).

DISCUSSION

From our experience with the Sardinian population, herein reported, we may draw several conclusions. First of all, carrier screening, counseling, and prenatal diagnosis appear to be effective means of controlling an inherited recessive disorder such as β-thalassemia at the population level. This experience may be taken as a

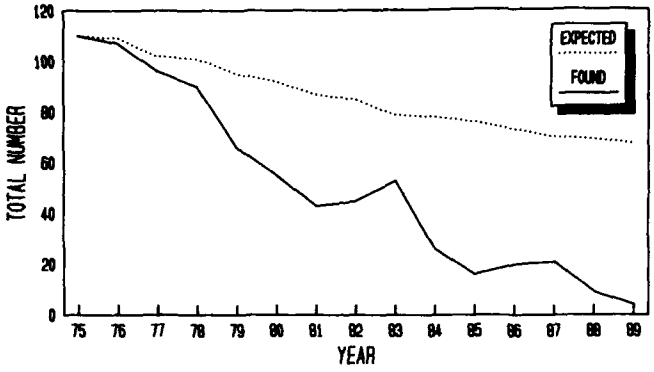

FIGURE 3. Fall in the birth rate of babies with homozygous β-thalassemia in Sardinia. Expected values are calculated on the basis of the carrier rate and the number of live births per year.

model for future genetic preventive programs for other recessive inherited disorders, such as, for instance, cystic fibrosis. Further improvement may be obtained by educating the population and especially by introducing the topic of thalassemia into the middle- and high-school curriculum. In fact, for the large majority of couples who were ignorant about thalassemia in our population, both parents were of very young age and finished attending school very early. Dot-blot analysis of amplified DNA with allele-specific probes is a very simple procedure for prenatal diagnosis. This procedure requires a very small amount of DNA—of the order of 5 μg—gives the results within 24 h from chorionic villi sampling, and can be done without the use of radioactive probes. However, even with careful dissection of chorionic villi from maternal decidua, we have shown that, especially with very small samples, in a limited number of cases co-amplification of maternal sequences can occur in such a

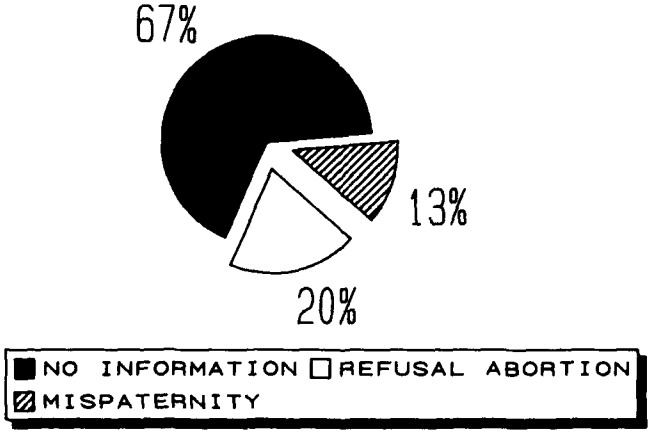

FIGURE 4. Residual births of babies with thalassemia major in Sardinia.

way as to cause misdiagnosis. To avoid this error, it seems necessary to monitor for the presence of co-amplified maternal sequences by the simultaneous amplification of a polymorphic DNA sequence.

Prenatal diagnosis is nowadays carried out in practically all cases by chorionic villi analysis because after genetic counseling, couples choose this procedure instead of amniocentesis.[20] The main reason for this decision is the earlier stage of gestation in which chorionic villi analysis may be carried out. For chorionic villi sampling, we use the transabdominal approach, which, in our experience, is associated with less risk of fetal loss as compared to the vaginal route. However, for a final conclusion on this point the results of a randomized study, now in progress, should be awaited.

One of the principles of genetic counseling is the accurate description of the natural history of the disease. Because the clinical phenotype of homozygous β-thalassemia may be heterogeneous, and a number of cases, referred to as thalassemia intermedia, may have a mild course not requiring transfusions, it will be useful, in order to improve the counseling, to be able to predict the clinical phenotype. However, in our population, prediction of the clinical phenotype on the basis of DNA analysis of prospective parents cannot be carried out, because the molecular bases of the mild forms of β-thalassemia in this population are for the vast majority of the cases not yet defined.[9] In other populations, however, finding very mild β-thalassemia mutations such as the β^{-101} (C→T), β^{-87} (C→G), or the β^{+} IVS-1, nt 6 in both or even in one parent may allow the prediction of a mild phenotype in the offspring.[21-24] It is well known that co-inheritance of α-thalassemia with homozygous β-thalassemia may result in a mild clinical phenotype.[25,26] However, this effect of α-thalassemia is not consistent, and, thus, the investigation of the presence of α-thalassemia in the prospective parents does not seem to be useful for improving the genetic counseling.[9]

Future development in this field will involve either the sampling procedure or the method of analysis. The possibility of defining the mutation by the analysis of a limited number of cells or even a single cell may pave the way to earlier diagnosis in the pregnancy or to preimplantation diagnosis. It is reasonable to assume, in fact, that the minimal amount of cells taken either by amniocentesis or chorionic villi sampling at 6–7 weeks gestation may be sufficient to yield enough DNA as a template for DNA amplification.[27] Preimplantation diagnosis may also be carried out by biopsy at the morula stage following *in vitro* fertilization[28] or by biopsy of the blastula washed out from the uterine cavity following *in vivo* fertilization.[29] From the technical point of view, further improvement, especially in terms of rapidity and simplicity, may be achieved by the use of reverse oligonucleotide hybridization, denaturing gradient gel electrophoresis,[30,31] or heteroduplex chemical mismatched cleavage analysis.[32,33] Prenatal diagnosis of β-thalassemia has greatly benefited the Sardinian population, both at the level of the individual, by avoiding the tragedy of a child affected by thalassemia major, and at the level of society, by avoiding the enormous financial burden created by such a disease. We do hope that in the near future in Sardinia, by further education of the population, thalassemia major may solely exist as an historical textbook description.

SUMMARY

This paper reviews the characteristics and the results of 15 years of experience with a preventive program, based on carrier screening and prenatal diagnosis, designed to control thalassemia major in the Sardinian population. The education of the population about thalassemia and the modalities for its prevention was accom-

plished via the mass media. Carrier screening was carried out voluntarily on couples of child-bearing age. Prenatal diagnosis was initially carried out by fetal blood analysis; since 1983, it has been done by DNA analysis on non-amplified or amplified DNA. Different chorionic villous sampling procedures have been used. Nowadays, we have adopted the transabdominal approach because, in our experience, it seems to be associated with a low risk (2%) of fetal mortality. At the present time, the β-thalassemia mutations are detected directly by dot-blot analysis of amplified DNA with ^{32}P- or horseradish peroxidase–labeled allele-specific oligonucleotide probes. Two oligonucleotide probes, one complementary to the codon-39 nonsense mutation, which accounts for 95.7% of the β-thalassemia chromosomes in the Sardinian population, and the other complementary to the frameshift at codon 6, which is the second most common mutation in our population (2.1%), allow us to make prenatal diagnosis in the large majority of cases. Notwithstanding a careful dissection of maternal decidua from chorionic villi, co-amplification of maternal sequence was detected in 4 out of 425 cases tested by this procedure. In order to avoid this pitfall, the simultaneous amplification of highly polymorphic VNTR (variable number of tandem repeats) segments could be used. On the whole we have so far carried out 2711 prenatal tests: 1130 by fetal blood analysis, 1156 by oligonucleotide hybridization on electrophoretically separated DNA fragments, and 425 by dot-blot analysis on amplified DNA with allele-specific oligonucleotide probes. Two errors occurred by fetal blood analysis and none by DNA analysis. The incidence of thalassemia major declined from 1:250 live births in the absence of prevention to 1:1000 after the establishment of this program, indicating that carrier screening and prenatal diagnosis are effective means for preventing thalassemia major at the population level.

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Prenatal Diagnosis of Thalassemia and of the Sickle Cell Syndromes in Greece[a]

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Prenatal diagnosis is the ultimate step in the process of prevention of inherited disease. Its availability encourages individuals at potential risk to report for screening and helps couples who are found to be at risk to make families with healthy children. This statement is now fully confirmed in Greece where prenatal diagnosis of thalassemia and sickle cell syndromes has been provided since 1977 to an ever-increasing number of couples at risk.

The present paper aims to review the evolution of this process over the years, to report the results of the Greek program, and to bring up questions which appear important for future planning both from the medical and from the administrative point of view. The paper will avoid reporting in detail data which have already been presented[1,2] or are of local interest only. In brief, Greece is a country with high endemicity of thalassemia (carrier frequency 7.5%) and several foci of sickle cell syndromes (mean frequency of hemoglobin S carriers of the order of 1%). The distribution of both genes is quite heterogeneous (FIG. 1). The number of affected babies expected to be born annually is estimated at roughly 150; this number is lower than those from earlier calculations because of the significant decrease of birthrate which occurred in Greece over the last decade. The number of patients actually

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surviving is of the order of 4,000. The impact of the latter on the socioeconomic system of the country is extremely severe, considering that all medical services, including one and one-half tons of desferrioxamine yearly, are provided to the patients virtually free of charge from the health authorities.

To prevent an accumulation of new cases, the Greek universities and, later on, the health authorities, the army medical services and other organizations have

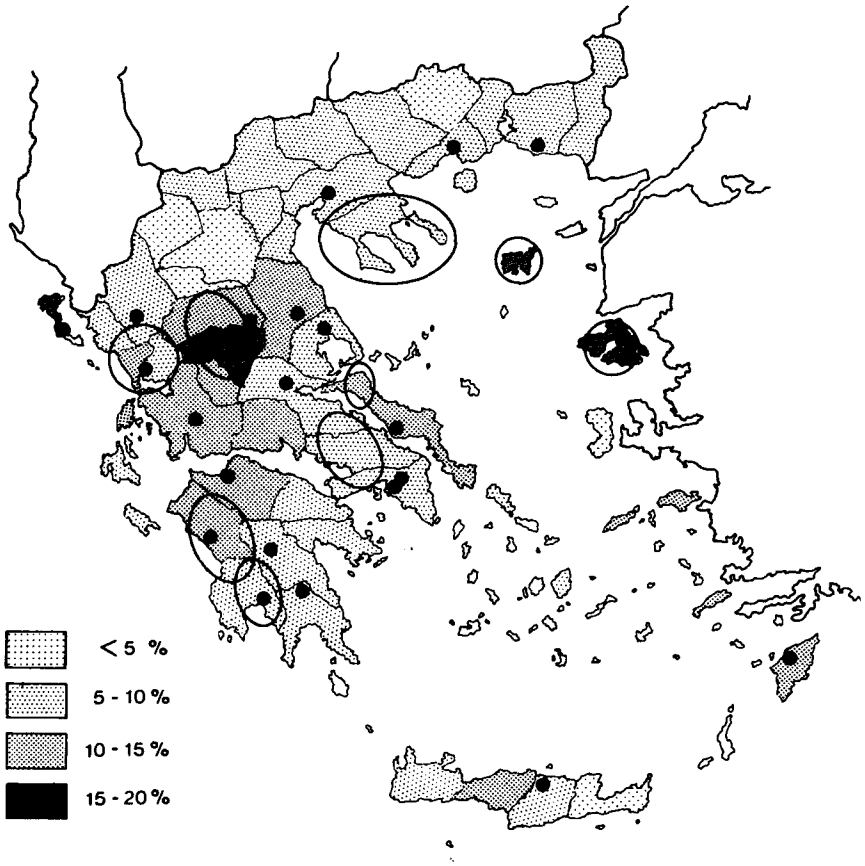

FIGURE 1. Map of Greece depicting the distribution of β-thalassemia carriers (*hatched areas;* percentages indicated on the map), the foci with a high frequency of Hb S (*circles*), and the "Units of Prevention" for carrier identification (*black dots*) in the main towns of the country.

provided the possibility of carrier identification to virtually all young people who request this examination. The national prevention program now provides this service through a number of "Units of Prevention" attached to hospitals of Athens and other towns of Greece, mainly in areas with high frequencies of the abnormal genes (FIG. 1). After carrier identification, couples in which both parents are heterozygotes are referred for counseling and prenatal diagnosis to our unit.

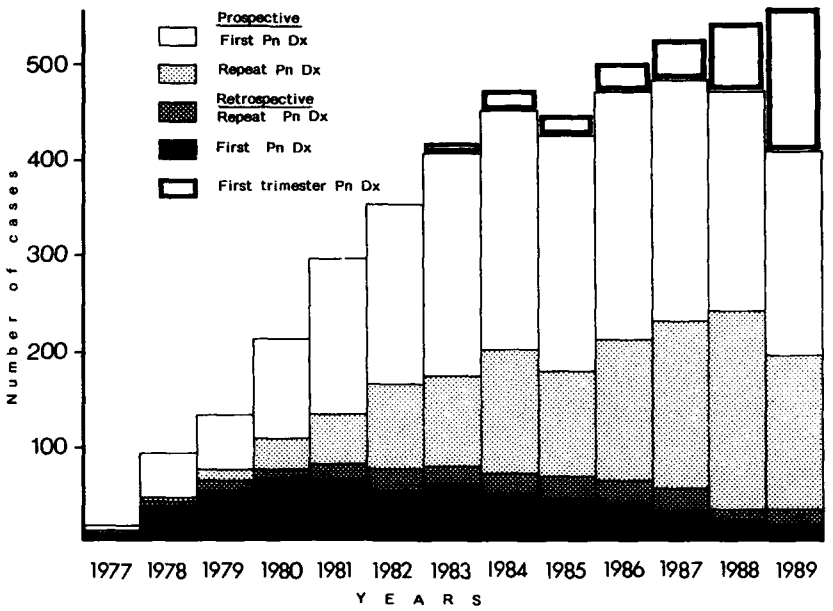

FIGURE 2. Mode of presentation to the prenatal diagnosis unit of couples at risk. Retrospective prenatal diagnosis (Pn Dx) has helped several couples with one or more affected children to obtain healthy siblings but is gradually diminishing. On the other hand, prospective diagnosis is increasing at a fast rate, both for the first attempt and for subsequent repeats. Also increasing fast is the number of women who seek prenatal diagnosis in the first trimester of pregnancy.

The mode of presentation of the couples who requested prenatal diagnosis over the last thirteen years is analyzed in FIGURE 2. Each yearly column indicates the number of first retrospective diagnoses, for couples who reported for prenatal diagnosis after the birth of a child with thalassemia major; the number of repeat retrospective diagnoses, for the same women who sought prenatal diagnosis twice or more; the number of first prospective diagnoses, for couples who came to us prior to having children; and the number of repeat prospective diagnoses, for the same women who had a repeat prenatal diagnosis, irrespective of the result of the first prospective diagnosis. In these cases, the test was carried out by fetal blood sampling. The rectangles above the columns represent cases of prenatal diagnosis with trophoblast studies, which gradually substituted for the original procedure. The total number of pregnancies examined so far is 4217. It is evident that, over the years, retrospective diagnoses have gradually diminished, while the prospective ones now constitute more than 90% of the total.

We consider that this observation reflects the level of sensitization of people, the ever-increasing collaboration of obstetricians, and the efficacy of the carrier identification system. Another point shown in FIGURE 2 is that the number of women who request a second or repeat prenatal test is also increasing. This finding indicates that the obstetrical procedure and dangers have been accepted by the women at risk and that they insist on having a well child, irrespective of the outcome of the preceding prenatal test. In fact, analysis of the data on women who requested prenatal diagnosis for a second or more times shows that 28% of these women had been given

a bad result and proceeded to selective abortion, 18% had aborted as an obstetrical complication, and 54% requested a repeat test after having had a good result with the first.

An analysis of the prenatal diagnoses carried in Greece over the last 13 years is presented in TABLES 1–4. Most couples at risk (TABLE 1) were typical high hemoglobin (Hb) A_2 carriers. Next in frequency come the pregnancies at risk for Hb S/β-thalassemia, then homozygous Hb S disease, and other indications present in a small minority of cases.

The obstetrical techniques initially applied were blind placental blood sampling and blood sampling from the placental vessels through a fetoscope. Over the last couple of years, the approach which is used almost exclusively is that of direct puncture of the umbilical veins with a long 21-gauge needle under ultrasound guidance. This technique provides 100% fetal red cell samples in almost all cases and has virtually eliminated the need for the painstaking and risky Orskov procedure.

Obstetrical complications leading to fetal loss are summarized in TABLE 2, which shows clearly that the overall risk of fetal loss following red blood sampling is high, with a mean percentage varying around 2.5%. However, it should be remembered that this toll reflects also the time required for the training of at least ten new obstetricians and for the development of the obstetrical techniques over the years.

In the laboratory, HPLC is gradually substituting for the conventional carboxymethyl cellulose (CMC) chromatography method.[3,4] Over the last two years, more than 50% of our pure fetal cell samples were examined by this procedure. The advantages of chain separation by HPLC are, in summary, that (1) the procedure is carried out on hemolysates and obviates the painstaking and messy preparation of globin; (2) it can be completed in only a couple of hours, in contrast to the 16-h globin chain separations on CMC; (3) it requires simpler and safer reagents, thus avoiding the disastrous effects of the ever-present urea in the CMC method; (4) it yields more accurate chain separations; and (5) it is significantly cheaper to run. Moreover, the fact that in most instances HPLC separations are carried out on pure fetal hemolysates allows direct confirmation of the presence of the expected amount of β^A chains by monitoring the optical density of the eluate at 215 nm. Disadvantages of HPLC include the considerably high initial investment to set up the system and the required maintenance and repair, which are often not available in developing countries.

Over the years, we have often considered the various modifications of the original

TABLE 1. Summary of Results of Prenatal Diagnosis of Thalassemia and the Hemoglobinopathies in Greece by Fetal Blood Studies, May 1977 through December 1989

Risk Combinations[a]	Diagnoses	
	n	%
β^{th}/β^{th}	3378	80.01
$\beta^{th}/\delta\beta^{th}$	130	3.09
$\delta\beta^{th}/\delta\beta^{th}$	11	0.26
β^{th}/β^{S}	494	11.73
β^{S}/β^{S}	83	1.97
$\delta\beta^{th}/\beta^{S}$	7	0.17
Severe α-thalassemia	16	0.38
Other	98	2.33
Total	4217	100.00

[a] β^{th}, β-thalassemia; β^{S}, sickle cell anemia.

TABLE 2. Prenatal Diagnosis of Thalassemia and the Hemoglobinopathies in Greece by Fetal Blood Studies, Annual Results for May 1977 through December 1989

Results	1977	1978	1979	1980	1981	1982	1983	1984	1985	1986	1987	1988	1989	Total
Fetal blood studies (*n*)	14	90	132	209	295	354	405	454	424	473	484	470	407	4211
Cases diagnosed to have thalassemia major														
n	1	24	25	48	95	110	106	103	123	122	111	101	99	1068
%	7.1	26.7	18.9	23.0	32.2	31.0	26.2	22.7	29.0	25.8	23.0	21.5	24.3	25.36
Cases expected to have thalassemia major (*n*)[a]	3.5	22.5	33.0	52.2	73.7	88.5	101.0	113.5	106.0	118.2	121.0	118.0	102.0	1053
False diagnoses														
False negative	1	1	3	3	2	1	2	0	0	1	0	1	1	16
False positive (probable)[b]	0	0	0	2	1	0	0	0	0	0	0	1	0	4
Obstetrical complications														
Failures to obtain sample[c]	0	2	8	5	4	1	1	4	4	3	1	—	—	33
Fetal losses directly associated with the procedure	4	10	7	10	9	9	8	10	12	4	6	6	4	99[d]

[a]Numbers based on expectation of 25%.
[b]May represent the result of samples being switched in the laboratory.
[c]Most of these failures were followed by fetal loss; a few others were lost to follow up.
[d]Approximately 2.5% of total procedures.

technique, aiming to make it even faster or applicable to samples containing only a few fetal cells. However, the continuous pressure of routine work in our laboratory has never allowed us time to experiment with them. To increase the safety of our results, we routinely avoid conveying them to the interested couples before confirming the presence of Hb A by isoelectric focusing of all pure fetal cell samples.[5,6] Our cut off level of β-chain synthesis by one normal gene at the 20th week of pregnancy is 3% of the total γ-chain synthesis.

FIGURE 3 confirms that the annual percentage of positive diagnoses did not deviate considerably from the anticipated 25%. However, false negative errors were not avoided. Although most of them are due to an over-optimistic interpretation of the β to γ ratios, four may have resulted from switching of samples during the

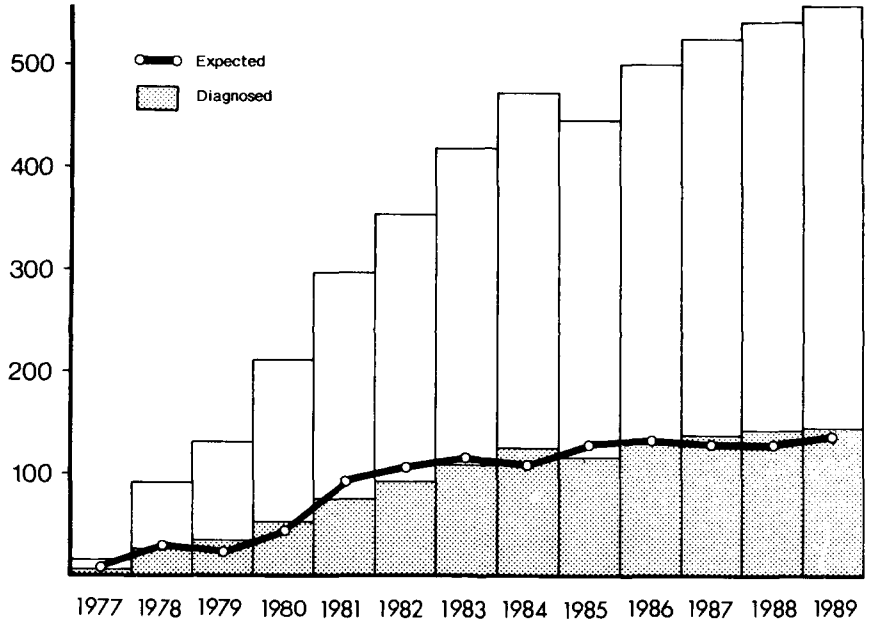

FIGURE 3. Comparison of the number of cases diagnosed as affected, using either fetal blood sampling or trophoblast DNA analysis, with the expected level of 25%.

multiple steps of the procedure and regrettably imply the existence of an equal number of false positive cases (TABLE 2).

Prenatal diagnosis by chorionic villi DNA analysis started in 1983. At the beginning it was restricted to pregnancies at risk for one or two β^S genes, because, for a long while, *Hpa* I was the only restriction enzyme we were able to obtain in Greece. The situation has changed since 1986, when, after long and tiring procedures, we managed to solve the logistic problems associated with this approach. The development of first trimester prenatal diagnosis over the last seven years is shown in FIGURE 1; its detailed analysis appears in TABLE 3.

The distribution of the normal and thalassemic β gene cluster haplotypes in Greece is given in TABLE 4. Micromapping did not disclose any foci of interest.

TABLE 3. Prenatal Diagnosis of Thalassemia and Hb S Syndromes by Chorionic Villi DNA Studies in Greece, 1983–1989

Results	1989[a]	Total
Cases (*n*)		
Total	139	321
Accepted with 100% feasibility	128	250
Accepted with 50% feasibility	11	71
The risk (*n*)		
Hb S/Hb S	3	36
Hb S/β-thalassemia	30	92
β-thalassemia/β-thalassemia	106	188
Severe α-thalassemia	0	3
Other	0	2
Failures to give results (*n*)	5	64
Successes (%)	97	80
Results of tests		
Normal or carrier (*n*)	101	195
Affected (*n*)	26	54
Affected (%)	25.7	27.2
Complications (*n*)		
Result followed by fetal loss	2	11
No result followed by fetal blood studies	4	49
No result followed by fetal loss	0	4
Lost to follow up	0	6

[a]Data for 1989 are given separately, since they are more representative of the present situation.

Moreover, there is no indication of linkage disequilibria, apart from those of the *Ava* II polymorphic site within the ψβ gene, which is usually absent when in association with haplotype I β-thalassemia. Most informative are the polymorphic *Hin*d III sites within the $^{G}\gamma$ and $^{A}\gamma$ genes, as well as the *Hin*c II site 5′ to the ε gene, especially when used in combination with the *Ava* II/ψβ site. Using this information, the feasibility of 100% prenatal diagnosis is no less than 80% for the families reporting for the test.

Availability of prenatal diagnosis through restriction fragment length polymorphism (RFLP) studies resulted in a rapidly increasing demand. However, given the prospective character of most requests, this created a serious problem for a while, since most couples did not have an affected child, necessitating extensive and costly family studies in order to reveal the most informative RFLPs, as well as a minimum time margin to complete the analysis. To confront the problem of the ever-increasing demand and to avoid the undesirable stress on the staff to provide answers to the couple in a very short time, our policy is to accept couples for prenatal diagnosis by DNA studies only when they are willing to wait to start the pregnancy until after completion of their β gene RFLP analysis.

According to a recent publication, these problems may be decreased by selective amplification of sequences flanking each polymorphic site and cleavage of the polymerase chain reaction (PCR) product with the appropriate enzymes, the end

result being one or two ethidium bromide–positive bands on a gel only a few hours after the DNA extraction and without the use of radioisotopes.[7] However, even this method is time consuming and risky, given the large number and extreme heterogeneity of the samples which have to be processed daily under pressure.

Of course, when using RFLPs, the danger of a false interpretation due to meiotic crossing-over is always present. However, luckily enough, this complication has not as yet occurred.

As presented in previous papers in this volume, it has become clear that the method of choice for the future is the direct identification of the thalassemic genes by the corresponding oligonucleotides. This technique has already been applied routinely in Sardinia for many years, but it could not be reproduced consistently in several other laboratories, most probably because oligonucleotides recognizing mutations other than the β^0 −39 C→T nonsense mutation do not work as well. The problem was solved with the introduction of gene amplification technology by PCR.[8,9] This procedure, now standardized and used in several laboratories all over the world,

TABLE 4. Frequency of the β Gene Cluster Haplotypes and of the β-Thalassemic Mutations in Greece

		Normal Chromosomes (%)	Chromosomes of Patients: Thalassemia Major (%)	Chromosomes of Patients: Thalassemia Intermedia (%)
Haplotypes[a]				
I		38.3	47.9	36.8
II		15.8	17.5	5.2
III		2.8	2.8	7.0
IV		1.9	0.9	—
V		12.3	12.0	5.2
VI		2.2	7.4	33.3
VII		4.4	4.6	7.0
VIII		—	—	—
IX		19.3	4.1	5.5
Other		3.0	2.8	—
Thalassemic mutations[b]				
IVS-1 nt 1	β^0		16.1	4.7
IVS-2 nt 1	β^0		2.3	3.5
FS codon 5	β^0		—	1.2
FS codon 6	β^0		2.3	1.2
FS codon 8	β^0		0.4	—
β −39	β^0		19.4	5.8
IVS-1 nt 5	β^+		—	—
IVS-1 nt 110	β^+		42.0	30.2
IVS-2 nt 745	β^+		1.4	4.7
−87	β^{++}		2.3	5.8
IVS-1 nt 6	β^{++}		11.5	33.7
−101	β^{++}		—	1.2
Unknown			2.3	8.1

[a] *n* = 316 for normal chromosomes, 217 for chromosomes of patients with thalassemia major, and 57 for those with thalassemia intermedia.

[b] n = 217 for chromosomes of patients with thalassemia major, and 86 for those with thalassemia intermedia. FS, frameshift.

can also be reliably applied to DNA extracted from amniotic cells, which can be obtained at any stage of pregnancy; hence, it eliminates the risks of fetal blood sampling, even when the women requests the test past the tenth week of pregnancy.

The distribution of the thalassemic genes over the Greek population is summarized in TABLE 4. Most frequent are the mutations IVS-1 nt 110 G→A, resulting in a β^+-thalassemia, and the mutation β^0 −39 C→T, causing complete inhibition of β chain synthesis. Unidentified mutations are rare but they do occur. Micromapping of the defects failed to disclose any specific clusters. Given these frequencies, the feasibility of prenatal diagnosis of thalassemia in Greece by testing with oligomers recognizing the four commonest mutations is estimated at 65.5%, while expansion of this set to eight mutations increases this probability to 82.5%.

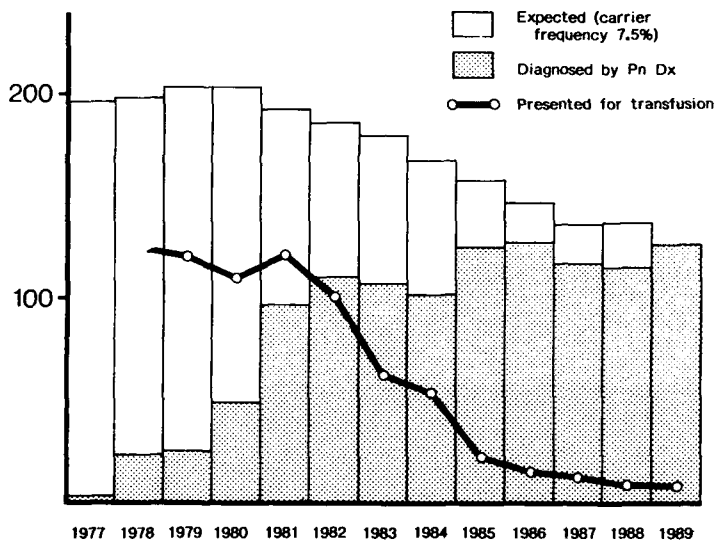

FIGURE 4. Efficacy of the program for the prevention of thalassemia in Greece over the last 12 years. The number of expected cases is based on a carrier frequency of 7.5%, evenly distributed over the country. Pn Dx, prenatal diagnosis.

Over the last year our laboratory has applied experimentally a set of non-radioactive oligomers kindly provided by the Cetus Corporation. The dot-blot patterns obtained were prominent and the background was completely clear. Whether these reagents will be widely available in the future we do not know. We must stress, however, that assays using non-radioactive oligomers represent an important advantage for laboratories such as ours or others further east because (1) they are faster, since the results can be available in no more than one day after the chorionic villi sampling; (2) they are safer compared to the use of radioactivity, a factor of great concern for the staff; (3) they are simpler, since the reactions can be developed in simple tanks at room temperature; and (4) they are significantly cheaper, if one takes into account not only the cost of the probes but also the important financial investment associated with the use of radioactive materials.[10]

More recent techniques, such as those involving denaturing gels,[11] and fluorescent[12] or allele-specific probes, are always of interest to us and have been evaluated on various occasions. However, for the time being, our main goal is to replace fetal blood sampling with molecular techniques as quickly as possible, a step which is not as simple as it appears, given the considerable work-load which has to be carried out daily with no intervals of time free for setting up new procedures.

FIGURE 4 is a graphic evaluation of the comprehensive program for the prevention of thalassemia in Greece over the years 1977–1989. Although, because of administrative inadequacies, the official registry of new cases did not work as expected, the information collected through personal contacts from all the major Units transfusing patients with thalassemia all over Greece shows that the annual number of new cases of thalassemia and compound Hb S syndromes has been varying around a value of ten over the last two or three years. In addition, analysis of the latter cases shows that only a couple of them were prenatal diagnostic errors, while several others came from parents who had "silent" or almost silent genes and had escaped carrier identification. In fact, retrospective blood tests revealed that in some instances the diagnosis was given on the basis of Hb A_2 determinations only (while the RBC indices had been ignored), while in others, the parents harbored the mild IVS-1 nt 6 T→C, the supermild −101 C→T, or other unidentified mutations.

The availability of prenatal diagnosis has been a key step in obtaining this satisfactory result. We acknowledge with thanks all the scientists who have contributed directly or indirectly in implementing this process and who are not cited here by name.

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Antenatal Diagnosis

Summary of Results[a]

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INTRODUCTION

Prenatal diagnosis of hematologic diseases began in 1974. A registry of cases tested worldwide has been maintained and published intermittently, encompassing June 1974–December 1978, January 1979–March 1980, April 1980–March 1981, April–December 1981, January–December 1982, January–December 1983, January 1984–December 1985, and the current Registry, January 1986–December 1989.[1-7] The data to be summarized here review the entire 15 years of these endeavors and represent a summary of the information provided by more than three dozen centers worldwide, involving more than a dozen countries (see TABLE 1 and ACKNOWLEDGMENTS). Twenty-three laboratories analyze fetal blood, 29 analyze DNA, and 3 obtain DNA samples from large numbers of cases but send them elsewhere for analysis. Sixteen centers deal with both blood and DNA specimens. This report will present the results of four categories of tests: fetal blood analyses for hemoglobinopathies, fetal DNA analyses for hemoglobinopathies, fetal blood analyses for other hematologic disorders, and fetal DNA analyses for other conditions, primarily hemophilias.

METHODS

Those centers of which the author was aware were sent a questionnaire to be filled in with their data. In some cases the centers provided data in their own format. Data were then collated using the LOTUS program. Data in the current registry, 1986–1989, are minimal estimates, since some centers may not have reported recently, some reports are incomplete, and some follow-up information, particularly regarding fetal losses and errors, was not always available. The data are reported according to the registry interval; data in the most recent registry, 1986–1989, are compared either with the data in each previous registry or with the cumulative data from 1974 through 1985.

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RESULTS AND DISCUSSION

The cumulative numbers of fetuses that have been studied for hemoglobinopathies or other diseases by blood or DNA sampling are shown in FIGURE 1. Of the close to 20,000 fetuses at risk for hemoglobinopathies, more than 13,000 had fetal

TABLE 1. Centers for Prenatal Diagnosis of Hemoglobinopathies Reporting for January 1986–December 1989

		Samples[a]	
Country	City	Blood	DNA
Australia	Melbourne	++	++
	Sydney	+	+
Canada	Calgary		+
	Hamilton		+
	Toronto		(+)
China	Beijing		+
	Guangzhou		+
	Nanning	+	+
	Shanghai		+
Cyprus	Lefkosa	+	
	Nicosia	+	+
England	London	++	(+)
	Oxford		+
France	Paris	++	+
Greece	Athens	+	+
Hong Kong	Hong Kong		+
Israel	Jerusalem	+	+
Italy	Cagliari	+	+
	Ferrara	+	+
	Milan	+	+
	Palermo	+	+
	Rome	+	
	Turin	+	+
Taiwan	Taipei	+	+
Thailand	Bangkok	+	+
Turkey	Ankara	+	
United States	Augusta		+
	Baltimore		+
	Cincinnati		+
	Framingham		+
	Honolulu		+
	New Haven	+	
	New York	+	+
	Rochester		(+)
	San Francisco		+

[a]Centers reported data through 1989. +, center analyzes indicated type of sample; (+), center collects indicated type of sample for analysis elsewhere; ++, 2 centers in the same city.

blood testing in the second trimester, and more than 6000 were tested using DNA, which was usually obtained in the first trimester. In addition, close to 5000 fetal blood samples were obtained for indications other than hemoglobinopathies, and more than 500 DNA samples also were obtained for other indications.

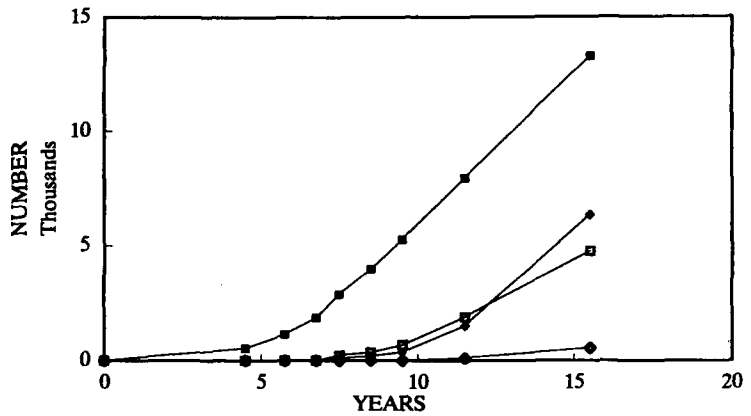

FIGURE 1. Cumulative number of cases examined prenatally for hematologic disease. Samples analyzed were (■) blood for the diagnosis of hemoglobinopathies, (◆) DNA for hemoglobinopathies, (□) blood for other diseases, (◇) DNA for other diseases.

FIGURE 2 shows the average number of cases studied per month for each registry interval. Fetal blood sampling for hemoglobinopathies has plateaued at more than 110 cases per month in each interval since 1981. DNA analyses for hemoglobinopathies are increasing and are now averaging 100 per month, close to the number of blood studies. Fetal blood testing for other diseases is at approximately 60 per month, while DNA testing for hemophilia is also on the rise.

Hemoglobinopathies were initially the only reason for fetal blood sampling, but they now comprise 65% of the blood samples (other risks will be discussed below). Similarly, hemoglobinopathies were at first the only hematologic indication for DNA sampling, and they still comprise more than 90% of the DNA specimens obtained for hematologic diagnosis.

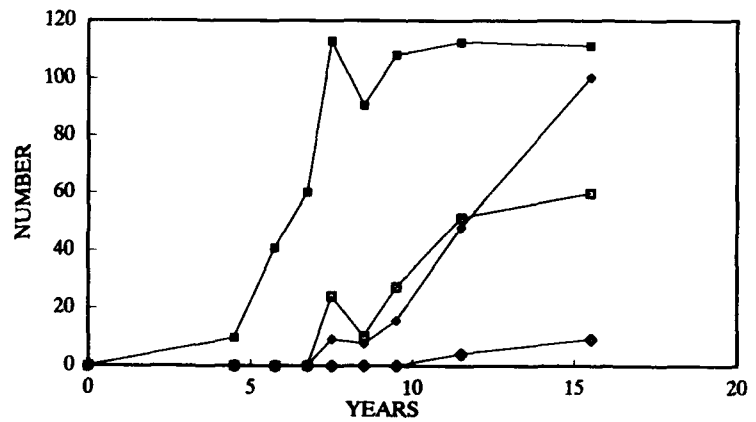

FIGURE 2. Average number of cases examined by prenatal diagnosis, per month for each registry interval: 6/74–12/78, 1/79–3/80, 4/80–3/81, 4/81–12/81, 1/82–12/82, 1/83–12/83, 1/84–12/85, and 1/86–12/89. Symbols as in FIGURE 1.

Over the period of time that blood sampling has been used for fetuses at risk for hemoglobinopathies, the proportion at risk for thalassemia increased from 90% in the early registries to more than 95% in the most recent one (TABLE 2). The rest were at risk for sickle cell disorders. Initially, thalassemia was the risk for only 30–40% of the hemoglobinopathy cases for which DNA sampling was performed; but DNA sampling for thalassemia now comprises more than 70% of the DNA testing for hemoglobinopathies, reflecting the ever-increasing ease with which thalassemia can be diagnosed using the tools of molecular biology (TABLE 3). From 1974 to 1981, DNA sampling was done for 40% of those at risk for sickle cell disorders but for only 3% of those at risk for thalassemia, and for less than 10% of the total group at risk for hemoglobinopathies (FIG. 3). The proportions tested by DNA sampling increased for each of the groups in each subsequent registry interval. In the most recent interval, 1986–1989, close to 50% of all cases were studied by DNA sampling,

TABLE 2. Fetal Blood Testing for Hemoglobinopathies: Fetuses at Risk, June 1974–December 1989

	6/74–12/85		1/86–12/89		Total 6/74–12/89	
Disorder	*n*	%[a]	*n*	%[a]	*n*	%[a]
Total cases	7955		5336		13291	
Thalassemia						
Total	7382	92.8	5159	96.7	12541	94.4
β	7303	98.9	5080	98.5	12383	98.7
α	8	0.1	14	0.3	22	0.2
δβ	10	0.1	14	0.3	24	0.2
αβ	10	0.1	4	0.1	14	0.1
E/β-thal	12	0.2	34	0.7	46	0.4
Lepore/β-thal	39	0.5	13	0.3	52	0.4
Sickle cell						
Total	573	7.2	177	3.3	750	5.6
SS	367	64.0	141	79.7	508	67.7
S/β-thal	198	34.6	29	16.4	227	30.3
SC	7	1.2	2	1.1	9	1.2
S/O-Arab	1	0.2	5	2.8	6	0.8

[a]Thalassemia and sickle cell disorders are calculated as % of total cases. Subgroups are calculated as % of total cases in their respective group.

including approximately 90% of those at risk for sickle cell disorders and 40% of the much larger number at risk for thalassemia.

The subgroups of risk categories for those studied by fetal blood sampling are shown in TABLE 2. In the entire 15-year interval, 94% of the fetuses were tested because they were at risk for thalassemia. More than 98% of the thalassemia cases were at risk for β-thalassemia, and less than 0.5% each were at risk for α-, δβ-, combined α- and β-, Hb E/β-, or Lepore/β-thalassemia. Amongst the 6% tested by blood sampling for risks of sickle cell disorders, overall close to 70% were for homozygous sickle cell anemia (SS), 30% for S/β-thalassemia, and a small percentage for SC, S/O-Arab, or other mixtures. These proportions were essentially similar at all intervals analyzed.

TABLE 3 shows the subgroups of the risk categories for those studied by DNA sampling. Approximately 70% were at risk for thalassemia, and 30% for sickle cell

TABLE 3. Fetal DNA Testing for Hemoglobinopathies: Fetuses at Risk, June 1974–December 1989

Disorder	6/74–12/85		1/86–12/89		Total 6/74–12/89	
	n	%[a]	*n*	%[a]	*n*	%[a]
Total cases	1511		4813		6324	
Thalassemia						
Total	823	54.5	3477	72.2	4300	68.0
β	719	87.4	2806	80.7	3525	82.0
α	93	11.3	527	15.2	620	14.4
δβ	2	0.2	0	0.0	2	0.0
Other	9	1.1	144	4.1	153	3.6
Sickle cell						
Total	688	45.5	1336	27.8	2024	32.0
SS	631	91.7	1178	88.2	1809	89.4
S/β-thal	16	2.3	106	7.9	122	6.0
SC	38	5.5	42	3.1	80	4.0
S/other	3	0.4	10	0.7	13	0.6

[a]Thalassemia and sickle cell disorders are calculated as % of total cases. Subgroups are calculated as % of total cases in their respective group.

disorders. In the entire time period, those at risk for β-thalassemia comprised more than 80% of the group at risk for thalassemia, α-thalassemia 15%, and other risks less than 5%. Amongst the group at risk for sickle cell disorders, 90% were for SS, 6% for S/β-thalassemia, and less than 5% for SC and other combinations. Testing for α-thalassemia was usually done by DNA sampling rather than by fetal blood sampling because of the ease of detection of this disease by the DNA method, due to the gene deletion.

The trends in methods for sampling fetal blood are examined in TABLE 4.

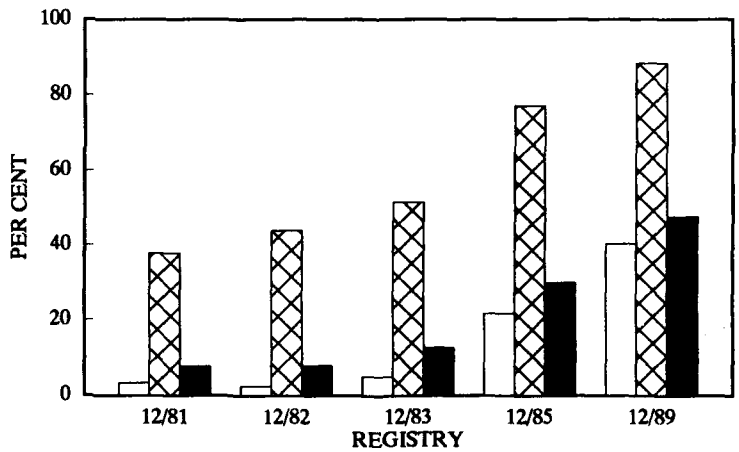

FIGURE 3. Proportion of fetuses at risk for the indicated hemoglobinopathy studied by prenatal DNA sampling in each registry interval. (*Open bars*) thalassemia, (*cross-hatched bars*) sickle cell disorders, (*solid bars*) total hemoglobinopathies.

TABLE 4. Fetal Blood Testing for Hemoglobinopathies: Methods and Results, June 1974–December 1989

Parameter	6/74–12/85		1/86–12/89[a]		Total 6/74–12/89[a]	
	n	%[b]	*n*	%[b]	*n*	%[b]
Total cases at risk	7955		5336		13291	
Thalassemia	7382	92.8	5159	96.7	12541	94.4
Sickle cell	573	7.2	117	3.3	750	5.6
Affected cases	2036	25.6	1273	23.9	3309	24.9
Thalassemia	1864	25.3	1245	24.1	3109	24.8
Sickle cell	172	30.0	28	15.8	200	26.7
Pregnancies continued	21	1.0	3	0.2	24	0.7
Thalassemia	14	0.8	2	0.2	16	0.5
Sickle cell	7	4.1	1	3.6	8	4.0
Fetal losses	303	3.8	105	2.0	408	3.1
Diagnostic errors	46	0.6	17	0.3	63	0.5
Sampling methods						
Total	7631		5336		12967	
Fetoscopy	5445	71.4	1236	23.2	6681	51.5
Aspiration	2175	28.5	69	1.3	2244	17.3
Cordocentesis	11	0.1	4031	75.5	4042	31.2
Analysis methods[c]						
Total	7955		5336		13291	
CMC	7455	93.7	2805	52.6	10260	77.2
HPLC	500	6.3	2210	41.4	2710	20.4
IEF			321	6.0	321	2.4

[a]Numbers in 1986–1989 may be low due to incomplete reporting/follow-up.

[b]Affected are calculated as % of total cases. Subgroups are calculated as % of total cases in their respective group. Pregnancies continued are calculated as % of affected cases. Losses and errors are calculated as % of total cases.

[c]Methods for sampling or analysis not always provided. CMC, carboxymethyl cellulose chromatography; HPLC, high pressure liquid chromatography; IEF, isoelectric focusing.

Initially, placental aspiration and fetoscopy were used, but almost all centers have now switched to cordocentesis (also known as percutaneous umbilical blood sampling, or PUBS), which is ultrasound-directed umbilical cord puncture without need for the direct visualization required for fetoscopy.[8] Another advantage of cord blood sampling is that the specimens are always pure fetal blood, and the problem of contamination with maternal blood, which plagued many in the early days, is now passé.

There have also been changes in the methods for analyses of fetal blood (TABLE 4). Until recently, globin chain synthesis was examined by labeling reticulocytes with radioactive amino acids, followed by separation of the globin proteins using carboxymethyl cellulose chromatography (CMC).[9] In the past 5 years, high pressure liquid chromatography (HPLC) has been used to separate small amounts of radioactive or non-radioactive proteins. HPLC provides an answer within a few hours, rather than the 2 day minimum needed for CMC columns. HPLC also separates β^E much more easily than do CMC columns.[10] Most recently, since cordocentesis samples now provide pure fetal blood, simple electrophoresis or isoelectric focusing (IEF) of hemoglobin tetramers has been used, without the need for radioactivity and biosynthesis.[11]

The trends in methods for obtaining DNA samples are outlined in TABLE 5. Amniocentesis at 16 weeks gestation prevailed until 5 years ago, when sampling of

chorionic villi (CVS) at 9–12 weeks became the method of choice.[12] CVS is done earlier in pregnancy than amniocentesis and thus provides obvious advantages to both patient and physician. Although the risk of fetal loss from CVS done for all genetic reasons is still approximately 4%, it is apparently less in the restricted context of the hemoglobinopathy patients (see below).

The types of DNA analyses are also shown in TABLE 5. The first method involved restriction fragment length polymorphisms (RFLP), but the use of this has declined with time.[13] In the most recent period reported, RFLP comprised less than 20% of the analyses. Other assays include the use of enzymes specific for the mutation of interest, such as *Mst* II for the β^S mutation of the sickle gene.[14] Some diagnoses are made based on gene deletion, such as those for α- or δβ-thalassemia.[15] Many samples are now amplified by the polymerase chain reaction (PCR)[16] and then examined using specific enzymes or using olignucleotides specific for the normal and mutant genes of interest.[17] This is the method of choice when the ethnic background of the parents indicates that a small group of mutations is possible. In a few recent cases, the group at Johns Hopkins School of Medicine (Baltimore) has used PCR to amplify relevant regions, followed by microsequencing. The combination of PCR and specific

TABLE 5. Fetal DNA Testing for Hemoglobinopathies: Methods and Results, June 1974–December 1989

	6/74–12/85		1/86–12/89[a]		Total 6/74–12/89[a]	
Parameter	*n*	%[b]	*n*	%[b]	*n*	%[b]
Total cases at risk	1511		4813		6324	
Thalassemia	823	54.5	3477	72.2	4300	68.0
Sickle cell	688	45.5	1336	27.8	2024	32.0
Affected cases	372	24.6	1197	24.9	1569	24.8
Thalassemia	195	23.7	879	25.3	1074	25.0
Sickle cell	177	25.7	318	23.8	495	24.5
Pregnancies continued	66	17.7	64	5.3	130	8.5
Thalassemia	3	1.5	26	3.0	29	2.7
Sickle cell	63	35.6	38	11.9	101	20.4
Fetal losses	30	2.0	47	1.0	77	1.2
Diagnostic errors	5	0.3	24	0.5	29	0.5
Sampling methods[c]						
Total	990		4813		5803	
Amniocentesis	440	44.4	782	16.2	1222	21.1
CVS	550	55.6	4031	83.8	4581	78.9
Analysis methods[c]						
Total	1251		1956		3207	
RFLP	354	28.3	335	17.1	689	21.5
Deletion	102	8.2	273	14.0	375	11.7
Enzyme	403	32.2	451	23.1	854	26.6
Oligo	392	31.3	223	11.4	615	19.2
PCR			669	34.2	669	20.9
Sequence			5	0.3	5	0.2

[a]Numbers in 1986–1989 may be low due to incomplete reporting/follow-up.

[b]Affected are calculated as % of total cases. Subgroups are calculated as % of total cases in their respective group. Pregnancies continued are calculated as % of affected cases. Losses and errors are calculated as % of total cases.

[c]Methods for sampling or analysis not always provided. CVS, chorionic villus sampling; RFLP, restriction fragment length polymorphism; Oligo, oligonucleotide; PCR, polymerase chain reaction.

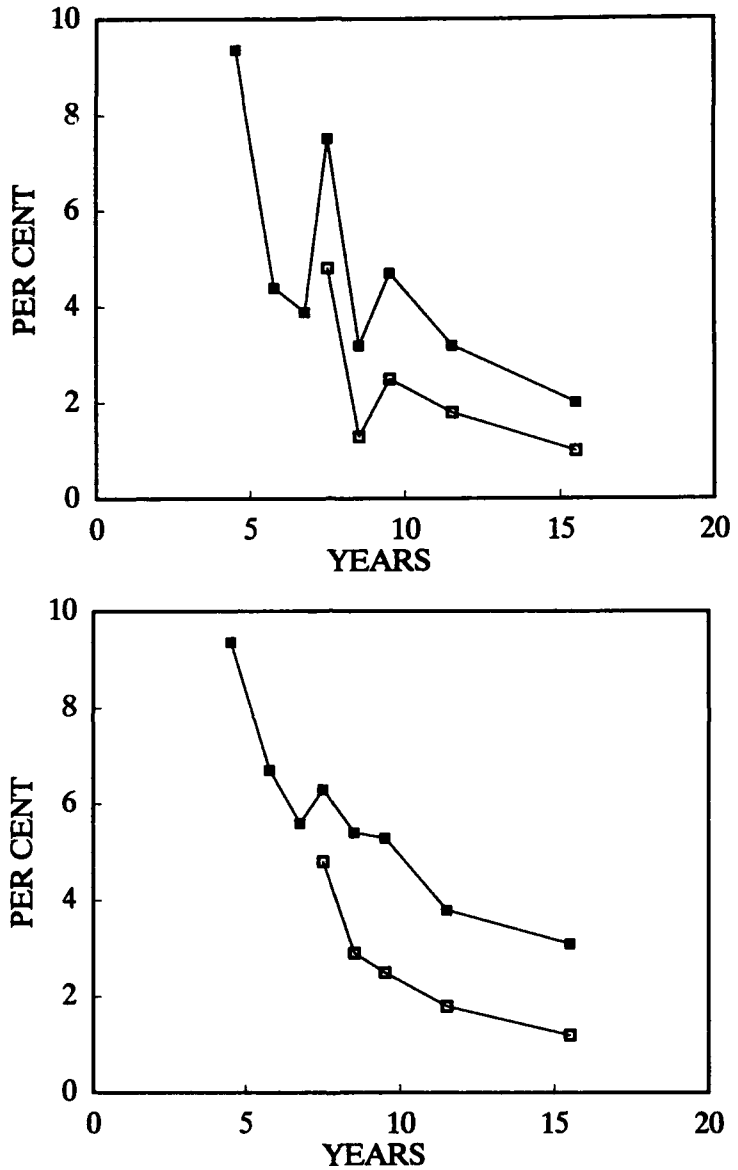

FIGURE 4. Fetal loss rate as percentage of cases examined for hemoglobinopathies. (**Upper panel**) calculated for each registry interval, (**lower panel**) cumulative data. (■) Blood, (□) DNA.

enzymes or oligonucleotides is probably the method most frequently used at this time, however.

The fetal loss rates from blood and DNA sampling are shown in FIGURE 4. The top panel shows the loss rates calculated for each registry interval, and the bottom

panel the cumulative rates. Basically, these results reflect a "learning curve." The loss rate from fetal blood sampling is now essentially 2%, and from DNA sampling, which is predominantly by CVS, close to 1%. These figures may be underestimates, due to incomplete follow-up.

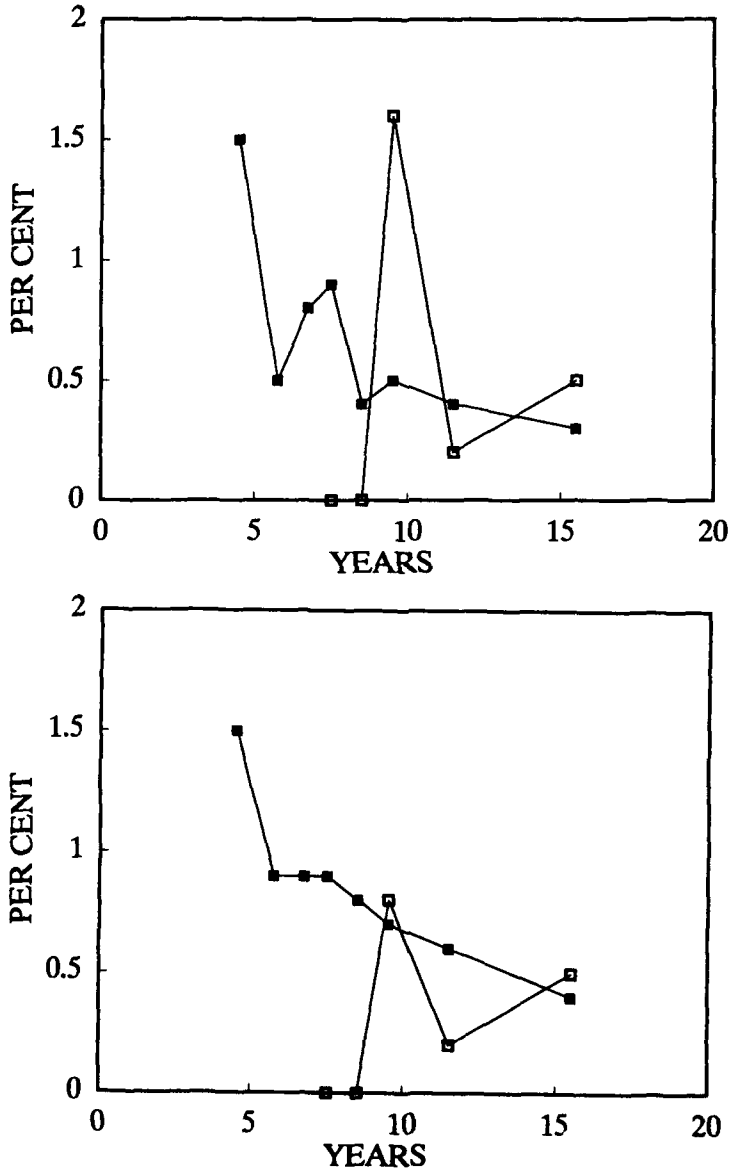

FIGURE 5. Diagnostic error rate as a percentage of cases examined for hemoglobinopathies. (**Upper panel**) calculated for each registry interval, (**lower panel**) cumulative data. (■) Blood, (□) DNA.

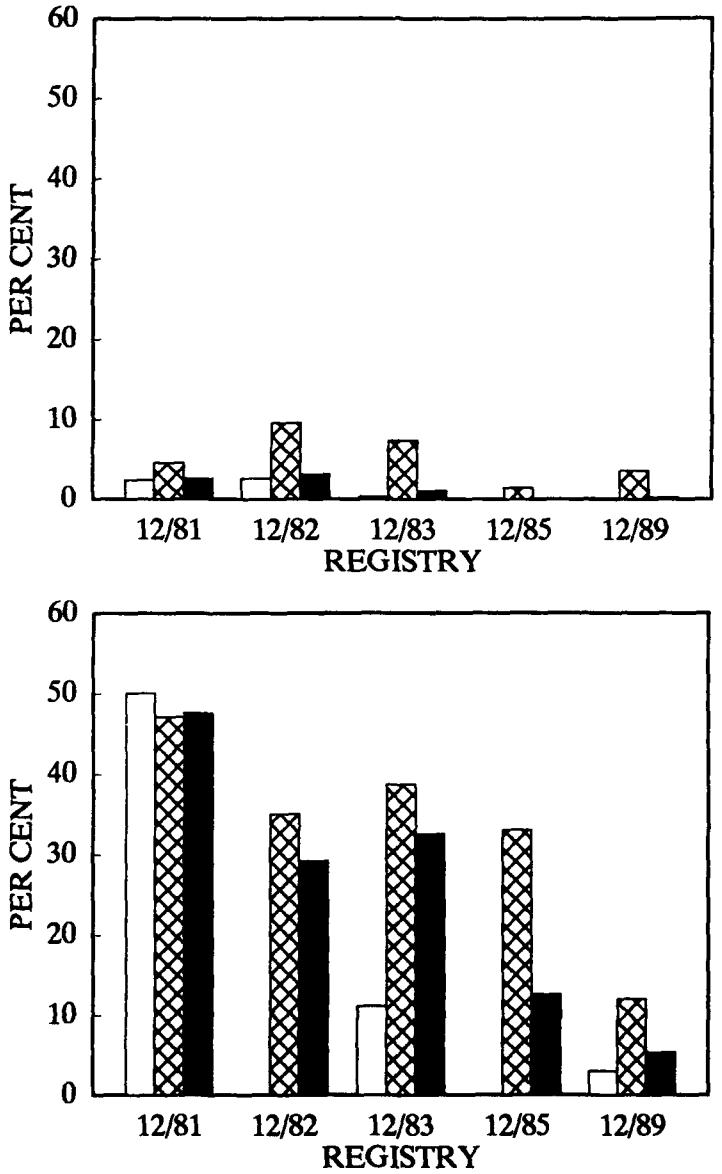

FIGURE 6. The proportion of pregnancies which were continued after diagnosis as affected with a hemoglobinopathy. (**Upper panel**) Diagnosis by fetal blood sampling, (**lower panel**) diagnosis by DNA sampling. (*Open bars*) diagnosis of thalassemia, (*cross-hatched bars*) sickle cell disorders, (*solid bars*) totals for all hemoglobinopathies.

The diagnostic error rates are shown in FIGURE 5, with rates calculated for each registry interval on top, and cumulative data on the bottom. Errors in analyses of both blood and DNA are now reported to be approximately 0.5 percent. Although these data may also suffer from incomplete follow-up, we tend to think that centers will be notified when they have made a misdiagnosis.

FIGURE 6 depicts the outcome of pregnancies diagnosed as affected. Blood samples are shown in the top panel, and DNA samples in the bottom panel. Generally, pregnancies in which the fetus was diagnosed as having a sickle cell

TABLE 6. Fetal Blood Testing for Other Diseases: Results, June 1974–December 1989

Parameter	6/74–12/85		1/86–12/89		Total 6/74–12/89	
	n	%[a]	*n*	%[a]	*n*	%[a]
Total cases at risk	1894		2869		4763	
Coagulation disorders[b]						
Total	564	29.8	278	9.7	842	17.7
VIII	473	83.9	145	52.2	618	73.4
IX	46	8.2	19	6.8	65	7.7
VWF	8	1.4	7	2.5	15	1.8
Other	37	6.6	107	38.5	144	17.1
Infection (total)	763	40.3	1278	44.7	2041	42.9
WBC disorders[c]						
Total	367	19.4	954	33.3	1321	27.7
CGD	7	1.9	4	0.4	11	0.8
Immunodeficiency	25	6.8	7	0.7	32	2.4
Neutropenia	2	0.5	0	0.0	2	0.2
Chromosomes	333	90.7	943	98.8	1276	96.6
RBC disorders[d]						
Total	157	8.3	266	9.3	423	8.9
Rh	146	93.0	249	93.6	395	93.4
Other	11	7.0	17	6.4	28	6.6
Miscellaneous	43	2.3	93	3.2	136	2.9
Fetal losses	60	3.2	4	0.1	64	1.3
Diagnostic errors	10	0.5	0	0.0	10	0.2

[a]Totals for each group are calculated as % of total cases. Subgroups are calculated as % of total cases in their respective group. Losses and errors are calculated as % of total cases.

[b]VIII, IX, and VWF: factor VIII, factor IX, and von Willebrand factor deficiency, respectively. Other: mostly platelet disorders.

[c]WBC: white blood cells. CGD: Chronic granulomatous disease. Chromosomes: cytogenetic studies for karyotypic abnormalities.

[d]RBC: red blood cells. Rh: hemolytic anemia due to Rh incompatibility. Other: enzyme deficiencies, membrane defects, anemias.

disorder were more likely than those with thalassemia to be continued. This occurred in up to 10% of those diagnosed by blood sampling and in approximately 40% of those diagnosed by DNA sample. Again, the values for the most recent interval undoubtedly suffer from a lack of follow-up data. In general, we all know that counseling for thalassemia major is more clearcut than it is for sickle cell anemia, since the latter condition has a much broader clinical variation than does Cooley's anemia. This is reflected in the consistently higher rates for continuation of the pregnancy in the sickle disorder groups.

TABLE 7. Fetal DNA Testing for Other Diseases: Results, June 1974–December 1989

Parameter	6/74–12/85		1/86–12/89		Total 6/74–12/89	
	n	%[a]	*n*	%[a]	*n*	%[a]
Coagulation[b]						
Total	96		348		444	
VIII	91	94.8	329	94.5	420	94.6
IX	5	5.2	19	5.5	24	5.4
Affected	17	17.7	94	27.0	111	25.0
Pregnancies continued	1	5.9	6	6.4	7	6.3
Fetal losses	1	1.0	0	0.0	1	0.2
Diagnostic errors	1	1.0	3	0.9	4	0.9

[a]Pregnancies continued are calculated as % of affected cases. Losses and errors are calculated as % of total.
[b]VIII, IX: factor VIII, factor IX deficiency, respectively.

The results of all of the fetal blood analyses reported for hemoglobinopathies are outlined in TABLE 4. Overall, approximately 25% of the more than 13,000 fetuses were diagnosed as affected, with an overall reported fetal loss rate of 3%, and a diagnostic error rate of 0.5%. Samplings are now done by cordocentesis, and analyses are shifting over to HPLC or IEF. Comparison of the current registry with the previous data shows that rates of fetal loss and diagnostic error improved with increasing numbers of cases.

TABLE 5 summarizes the results of all the fetal DNA testing. Again, close to the expected value of 25% of more than 6000 cases were diagnosed as affected, with a cumulative reported fetal loss rate of approximately 1% and a diagnostic error rate of 0.5%. Sampling is now primarily by CVS, unless the pregnancy is too advanced. Analyses use PCR and specific enzymes, oligonucleotides, or deletions.

TABLE 8. Fetal Monitoring for Hematologic Diseases: Summary, June 1974–December 1989

Parameter	6/74–12/85		1/86–12/89		Total 6/74–12/89	
	n	%[a]	*n*	%[a]	*n*	%[a]
Time interval (yr)	11.5		4		15.5	
Sample and diagnostic purpose						
Blood, hemoglobinopathy	7955	69.4	5336	39.7	13291	53.3
DNA, hemoglobinopathy	1511	13.2	4813	35.8	6324	25.4
Blood, other diseases	1894	16.5	2869	21.3	4763	19.1
DNA, other diseases	96	1.9	440	3.3	536	2.2
Diagnostic purpose						
Hemoglobinopathy	9466	82.6	10149	75.4	19615	78.7
Other diseases	1990	17.4	3309	24.6	5299	21.3
Sample type						
Blood	9849	86.0	8205	61.0	18054	72.5
DNA	1607	14.0	5253	39.0	6860	27.5
Total fetuses	11456		13458		24914	

[a]Values are calculated as % of total cases in that group.

TABLE 6 summarizes the use of fetal blood sampling for diagnoses other than hemoglobinopathies, which includes almost 5000 cases. Diagnosis of coagulation disorders such as hemophilias used to require fetal blood sampling but is now done using DNA samples. Diagnosis of platelet abnormalities still requires blood analyses. In Europe more than in America, blood sampling is needed to assess intrauterine infections such as toxoplasmosis, cytomegalovirus, or rubella. White blood cell analyses are done for a variety of reasons, including diagnosis of chronic granulomatous disease, immunodeficiencies and neutropenias, and particularly, for cytogenetic studies in fetuses at risk for karyotypic abnormalities. Red blood cell disorders diagnosed from fetal blood samples include Rh and other hemolytic anemias, non-anemic hydrops fetalis, and enzyme deficiencies. The fetal loss and diagnostic error rates for these cases resemble those for hemoglobinopathy blood cases.

The results of more than 400 DNA studies of fetuses at risk for hemophilias are summarized in TABLE 7. The proportion of affected cases is 25%, below the 50% expected for X-linked conditions. This is due to the inclusion in the testing of fetuses in which the sex was unknown until the DNA sampling was performed. It also includes cases in which the hemophilia was apparently a new mutation in the propositus. Six percent of the affected males were carried to term. The fetal loss and diagnostic error rates were both below 1%.

TABLE 8 summarizes the trend in prenatal diagnosis of hematologic diseases, which has been performed in close to 25,000 fetuses worldwide over a 15-year period. It must be repeated that current data do suffer from under-reporting and incomplete follow-up, probably indicating more strongly than anything else that these techniques are now functioning as diagnostic service procedures rather than as experimental research tests. There is a definite shift toward DNA analyses for all conditions and a trend toward examination of fetal blood for blood-borne diseases that are not hemoglobinopathies and may not even be hematologic (such as intrauterine infections and abnormal karyotypes). I think those who have been doing these analyses over the years should feel justifiably pleased that they have seen the immediate application of basic laboratory research to clinical needs and to public health issues worldwide.

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Antenatal Diagnosis

How to Deliver a Comprehensive Service in the United Kingdom

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FIGURE 1, which summarizes the aims of genetic screening and prenatal diagnosis, shows that the main objective is informed choice for couples at risk. To allow choice, one must offer screening and genetic counseling. Prenatal diagnosis is the approach chosen by most informed couples at risk of a severe genetic disorder. Divorce is not a common solution, and artificial insemination by donor is not popular.

In the United Kingdom, in principle, screening for hemoglobinopathy carriers, counseling, and prenatal diagnosis (by fetal blood sampling or chorionic villus sampling [CVS] and DNA analysis) is available to the whole population within the National Health Service. Our objective is to ensure that all couples at risk for having children with a hemoglobin disorder have the opportunity of informed choice. It is becoming increasingly clear that an outreach approach, including quality control, is needed to ensure that the service is delivered equitably to everyone who needs it.

Service monitoring in the Mediterranean region has shown for thalassemia that a comprehensive "control" program[d] can lead to near-eradication of the disease[1,2] (FIG. 2). For sickle cell disease we would expect a smaller reduction in the birth rate, since only 50% of counseled couples request prenatal diagnosis.[3,4]

In the United Kingdom, 6.5% of the population and about 10% of the births are in ethnic minority groups at risk for hemoglobinopathies. Thalassemias are carried by 3–17% of people of Mediterranean or Asian origin, and a sickle trait is carried by

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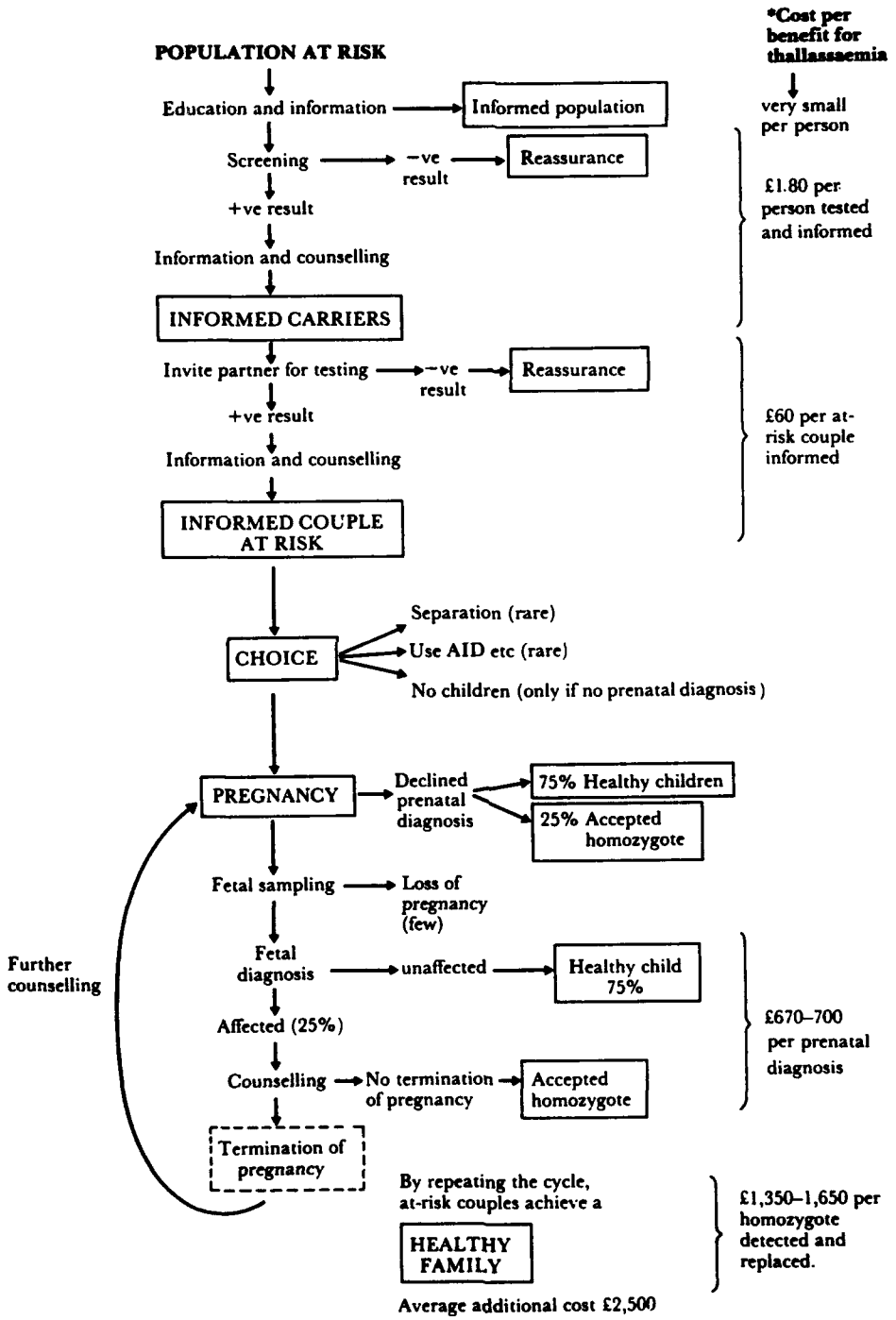
[d] The World Health Organization (WHO) defines a control program for an inherited disease as "a comprehensive strategy combining the best possible patient care with prevention based on community information, carrier screening and counselling, and the availability of prenatal diagnosis."[1]


*Cost per benefit for thallassaemia
POPULATION AT RISK
Education and information
Informed population
very small per person
Screening
−ve result
Reassurance
+ve result
£1.80 per person tested and informed
Information and counselling
INFORMED CARRIERS
Invite partner for testing
−ve result
Reassurance
+ve result
£60 per at-risk couple informed
Information and counselling
INFORMED COUPLE AT RISK
CHOICE
Separation (rare)
Use AID etc (rare)
No children (only if no prenatal diagnosis)
PREGNANCY
Declined prenatal diagnosis
75% Healthy children
25% Accepted homozygote
Fetal sampling
Loss of pregnancy (few)
Fetal diagnosis
unaffected
Healthy child 75%
Affected (25%)
£670–700 per prenatal diagnosis
Counselling
No termination of pregnancy
Accepted homozygote
Termination of pregnancy
Further counselling
By repeating the cycle, at-risk couples achieve a
HEALTHY FAMILY
£1,350–1,650 per homozygote detected and replaced.
Average additional cost £2,500

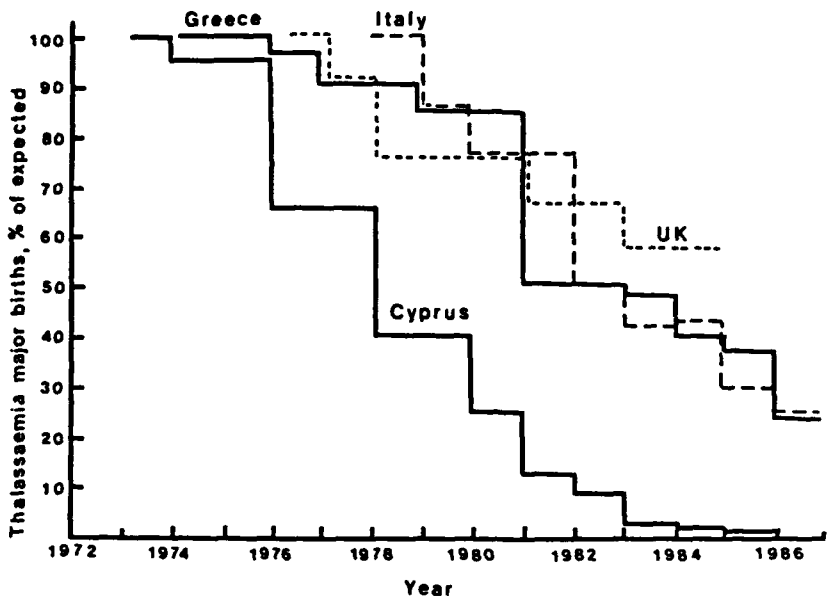


FIGURE 2. Change in birth rate of thalassemic children in four countries with the introduction of programs of education, screening, and prenatal diagnosis.

8%–25% of people of African or Afro-Caribbean origin. It is estimated that potentially about 150 infants with sickle cell disease and 60 infants with thalassemia major could be born annually in the United Kingdom. In fact, the number of thalassemia major births is much less than this; but in the absence of a national monitoring program, we do not know exactly how much less.

In this paper we have selected two points for discussion: (1) the question of how to deliver the screening, counseling, and prenatal diagnostic service to a Muslim ethnic minority and (2) the issue of misdiagnosis as a service becomes more widely disseminated.

FIGURE 3 (ethnic minority births as % of all births) shows that the populations at risk are concentrated in certain areas: e.g., 80% of British Cypriots live in London, and about 60% of British Pakistanis live in the north. In 1984, a survey of thalassemia major births showed that at that time the main impact of prenatal diagnosis was on Cypriots settled in London (80% reduction). The impact on Indians (30% reduction) was much smaller, and there had been very little impact indeed amongst Pakistanis

FIGURE 1. Flow chart summarizing some of the real costs and benefits of screening for prenatal diagnosis. Benefits are in *boxes*. It should be noted that birth of an accepted affected child (homozygote) to informed parents is counted as a benefit. The main cost, termination of pregnancy, is in a *dashed box*. For the sake of clarity, some costs, such as the consequences of false positive and false negative results in screening tests, have been omitted. The average financial cost/person for each step is indicated at the *right*. −ve, negative; +ve, positive; AID, artificial insemination by donor. (Reprinted from Ref. 12 with permission from the Royal College of Physicians.)

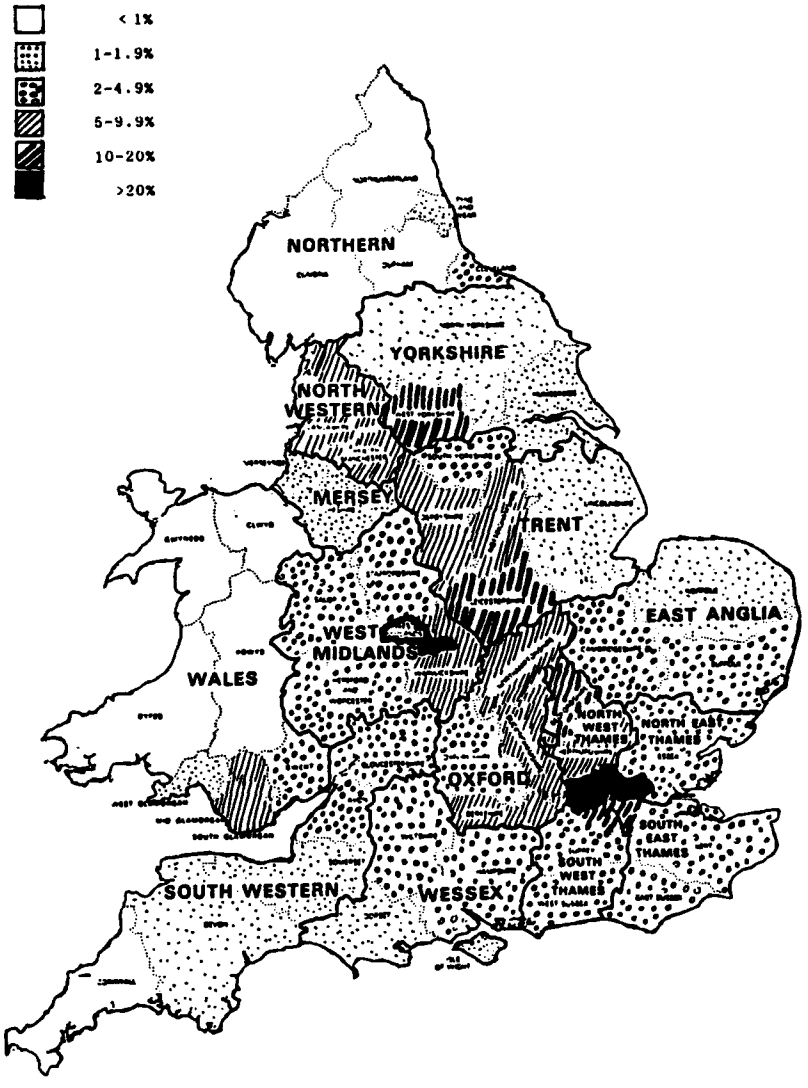


FIGURE 3. Births of ethnic minorities as % of all births in the United Kingdom, indicated for National Health Service regions.

(0% reduction).[5] Clearly, either prenatal diagnosis was not acceptable to most British Pakistanis or screening and genetic counseling was not being delivered appropriately to them. We had at that time already set up a study of the effect of thalassemia on the families in a city in the north of England with a sizeable British Pakistani community.

BRITISH PAKISTANIS AND PRENATAL DIAGNOSIS

In the city studied there are 18 families of Pakistani origin with thalassemic children. The researcher (A.D.) of this study, a woman of the same cultural, religious and linguistic background as these families, studied them in depth. British Pakistanis are Muslims, mostly first-generation migrants from a single rural area of Pakistan. Prior to this study, assumptions about their low utilization of prenatal diagnosis were based on social prejudice and influenced by language difficulties. Typical comments from health workers were "Muslim families have a fatalistic attitude and do not take any initiatives"; "they are not interested in prenatal diagnosis, as it is against their religion—there is no point in discussing it"; "they marry their cousins—If they didn't they wouldn't have genetic problems." The study was intended to answer three questions:

1. Had the parents been informed?
2. Had they understood?
3. What was their attitude to prenatal diagnosis?

Briefly, the answers were

1. They had been *told* about inheritance and prenatal diagnosis, but not in a way they could understand.
2. Only 1 of the 18 couples had really understood the inheritance of the disease and the availability of prenatal diagnosis.
3. They were not enthusiastic about mid-trimester prenatal diagnosis. However, they did not want to have thalassemic children and asked for earlier diagnosis. Some of the first couples to have a first-trimester prenatal diagnosis for thalassemia were from this group of families.[6]

FIGURE 4 summarizes the reproductive behavior of the families studied and their utilization of prenatal diagnosis, both before the study started and subsequently. The children in each family were divided into three groups, according to whether the pregnancy was before prenatal diagnosis was available; after prenatal diagnosis in the second trimester had become available, but before our study had started; or after the study was started and when first trimester prenatal diagnosis became available. The following paragraphs are summaries of each family's history.

Family 1. This family had two affected boys. The mother had a second-trimester prenatal diagnosis; the fetus was misdiagnosed as having thalassemia major, but the pregnancy was continued. This mother had not really wished to have prenatal diagnosis, but she underwent it to please her medical advisers. She is now in favor of prenatal diagnosis should her children need it.

Family 2. After having one thalassemic child, the mother referred herself for two mid-trimester prenatal diagnoses. The first abortion was by the outdated method of hysterotomy. The second abortion lasted two days because of uterine scarring. As a consequence of negative experiences, she rejected prenatal diagnosis and fortunately has had three healthy children.

Family 3. This family had not understood about the inheritance of the disease or prenatal diagnosis. When they did they had two prenatal diagnoses, despite the fact that the first one ended in the spontaneous abortion of an unaffected fetus.

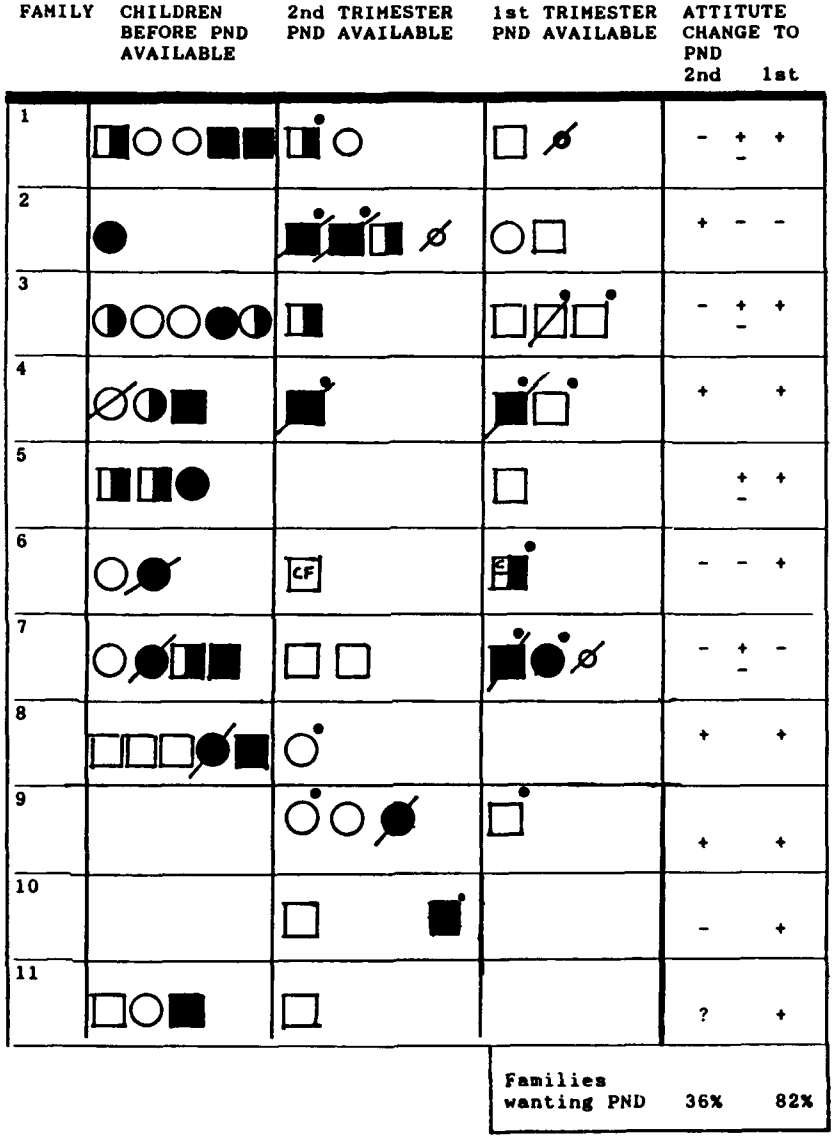


FIGURE 4. Summary of the reproductive behavior of 11 British Pakistani families in relation to information and the availability of prenatal diagnosis in the first/second trimester. The children in each family are divided into three groups: before prenatal diagnosis (PND) was available; after prenatal diagnosis in the second trimester had become available, but before our study had started; after the study was started and when first-trimester prenatal diagnosis became available. (□) Male, (○) female, (◨, ◑) heterozygotes, (■, ●) homozygotes: thalassemia major. CF, cystic fibrosis. *Small dot,* prenatal diagnosis performed.

Family 4. This was an educated family in which the father is an Imam of a minority Muslim sect. All three pregnancies following the birth of the affected son were undertaken only on condition that prenatal diagnosis was available.

Family 5. The couple had difficulty in accepting that their child had thalassemia major, since she had a milder disease than most of the other children with this disease. They did not request prenatal diagnosis partly for this reason. The affected child is now well, following a bone marrow transplant. The couple would request prenatal diagnosis in any subsequent pregnancies.

Family 6. The couple were transmitting both cystic fibrosis and thalassemia. When the study started the mother was completely against prenatal diagnosis, on religious grounds. However, the thalassemic child died following bone marrow transplantation. Prenatal diagnosis was requested in a subsequent pregnancy, in which the fetus proved to carry both thalassemia and cystic fibrosis.

Family 7. The parents are a very religious couple. They could not accept second-trimester prenatal diagnosis, but after the death of their thalassemic daughter, they requested prenatal diagnosis in two subsequent pregnancies. One was terminated. The second was also diagnosed as thalassemia major but was continued, and it was followed by a successful bone marrow transplant.

Family 8. This couple requested mid-trimester prenatal diagnosis. The fetus was unaffected.

Family 9. The parents were referred for prenatal diagnosis in the mid-trimester in their first pregnancy. They had not understood that the risk applied to all pregnancies. They would have wished to have prenatal diagnosis in the third pregnancy, which led to the birth of a thalassemic child who later died following a bone marrow transplantation. Prenatal diagnosis was requested in the first trimester in the fourth pregnancy.

Family 10. This couple were found to be at risk in their first pregnancy too late for prenatal diagnosis. They were informed of the possibility of first-trimester prenatal diagnosis and were interested. However, in their second pregnancy the general practitioner in charge of their case was not aware of the possibility of first-trimester diagnosis and did not refer them to the antenatal clinic until the 16th week. Mid-trimester prenatal diagnosis showed an affected fetus, but the couple felt that it was too late to terminate the pregnancy. They would have done so in the first trimester.

Family 11. The parents were aware of the inheritance of the disease and the availability of mid-trimester prenatal diagnosis. In their fourth pregnancy they asked for prenatal diagnosis to be arranged, but due to a misunderstanding they did not have it. The social worker telephoned a London hospital providing prenatal diagnosis to make arrangements. Because the hospital believed that the couple were resident in Pakistan, they informed her that prenatal diagnosis would cost the couple £600.00. They could not afford this and continued with the pregnancy unaware that a misunderstanding had occurred.

Thus, this study shows that many British Pakistani families have accepted prenatal diagnosis and that first-trimester prenatal diagnosis is more acceptable than

second-trimester prenatal diagnosis. The study clearly negates the notion that Muslims have no interest in prenatal diagnosis, and it underlines the importance of offering the service in a way that is appropriate to the community concerned.

CONSANGUINITY AMONGST BRITISH PAKISTANIS

Most of the couples studied were first cousins. The frequency of cousin marriage is falling among some populations, e.g., in Japan it has decreased from 13% in urban and 21% in rural areas to 2.9% in urban and 4.3% in rural areas since 1947.[7] By contrast, among British Pakistanis the frequency of close consanguineous marriage has increased, probably as a result of relative isolation. An inquiry among a 100 randomly selected British Pakistani mothers in the postnatal wards showed that 55% of young couples were first cousins, but only 33% of their mothers were married to first cousins.[8] This is consistent with a study in Lahore, Pakistan, in 1982, which showed 36% first-cousin marriages.[9]

This high frequency of consanguinity has led to some inappropriate reactions. For example, all 18 couples in our study had been told at least once by a health worker that their child's illness was due to their consanguinity. But the convention of consanguineous marriage is very widespread, involving over 17% of the world's population.[1] It was customary in biblical times, and it is considered to have important social functions especially protective to women.[10] The appropriate response is *not* to try to decrease the frequency of consanguineous marriage: there are no good genetic grounds for such a policy.[11] Rather, in such populations each index patient with a recessive-inherited disorder marks a family cluster at high risk. In principle it should be possible to deliver targeted genetic counseling very effectively in such populations by following up the extended family. This will need the help of trained community genetic counselors; though this idea is accepted[12] the service has yet to be developed.

MISDIAGNOSES

Unless awareness in the health service at large is improved, as services spread more widely there is an increasing risk of fetal misdiagnosis due to errors such as carrier misdiagnosis, misinterpretation of traits such as Hb Lepore, Hb D Punjab, etc. Prenatal diagnosis for the hemoglobinopathies has now been established for sixteen years in the United Kingdom, and considerable experience has accumulated both with mid-trimester prenatal diagnosis by fetal blood sampling and with first trimester prenatal diagnosis by CVS and DNA analysis.[6,13] Over the past five years our service at the University College Hospital has switched from largely mid-trimester to first-trimester prenatal diagnosis by CVS and DNA analysis (TABLE 1). Because of the complex population we serve, most DNA-based prenatal diagnoses are done by analysis of restriction fragment length polymorphisms (RFLPs). However, most couples coming for prenatal diagnosis do not have an affected child. In order to offer them CVS and DNA analysis, it is usually necessary to do extensive family studies; and even then DNA-based diagnosis is not possible in some cases.

One way of resolving this problem for couples who have an affected pregnancy terminated in the mid-trimester after fetal blood sampling is to use material from the placenta of that presumably homozygous fetus as the key to informative family studies. This usually allows prenatal diagnosis in the first trimester for such couples in the future: but the approach implies complete confidence in the diagnosis of the mid-trimester fetus.

TABLE 1. Number of Prenatal Diagnoses Done at University College Hospital

Year	Fetal Blood Sampling		CVS[a]
	n	Technique	*n*
1974–1981	137	Placentacentesis	
	329	Fetoscopy	
1982	81	Fetoscopy	5
1983	94	Fetoscopy	13
1984	48	Fetoscopy	37
1985	33	Fetoscopy	46
1986	42	Fetoscopy	69
1987	17	Fetoscopy (5); cordocentesis (12)	73
1988	22	Cordocentesis	75
1989	22	Cordocentesis	76
1990[b]	2	Cordocentesis	7
Total	827		401

[a]CVS, chorionic villus sampling.
[b]Values shown include data only through the end of January 1990.

At University College Hospital we have used this approach very cautiously, since globin biosynthesis using fetal blood is performed in the routine clinical diagnostic laboratory. This is not an ideal arrangement for a genetic service. When, in our opinion, an element of doubt existed in a diagnosis that led to termination of a

TABLE 2. Misdiagnoses in Globin Chain Biosynthesis Test

Case[a]	At Risk[b]	Diagnosis	Actual Result	Cause of Misdiagnosis
1974				
1.	Thal major	Unaffected	Thal major	Increase of β/γ ratio by γ mutant
2.	Thal major	Unaffected	Thal major	Range-finding[c]
3.	Thal major	Thal major	Thal trait	Patient refused repeat test; range finding[c]
4.	Thal major	Unaffected	Thal intermedia	IVS-1 nt 6/IVS-1 nt 6[d]
5.	Thal major	Unaffected	Thal intermedia	IVS-1 nt 6/IVS-1 nt 110[d]
1982				
6.	Thal major	Unaffected	Thal major	Bad elution pattern
7.	Thal major	Thal major	Probably unaffected	Samples switched
8.	Thal major	Unaffected	Thal major	Samples switched
9.	Thal major	Thal major	Thal trait[e]	Lab refused to repeat test[e]
10.	Thal major	Thal major	Thal trait[e]	Lab refused to repeat test[e]

[a]Service was run in a research laboratory in 1974 and in a routine clinical diagnostic laboratory from 1982.
[b]Thal, thalassemia.
[c]Range finding: laboratory was still in the process of establishing the β/γ range for a diagnosis of β^+-thalassemia major.
[d]The β/γ ratios obtained for cases 4 and 5 were well within the range for thalassemia trait, although the pregnancies resulted in children with thalassemia intermedia. The presence of the mild IVS-1 nucleotide (nt) 6 mutation, determined by genetic studies, explains this discrepancy.
[e]Pregnancies in cases 9 and 10 were terminated after diagnosis of thalassemia major in the fetuses by globin chain biosynthesis. Because of doubt about these results, our laboratory performed linkage studies in the extended family for each case and showed, by RFLP analysis, that the fetuses had thalassemia trait.

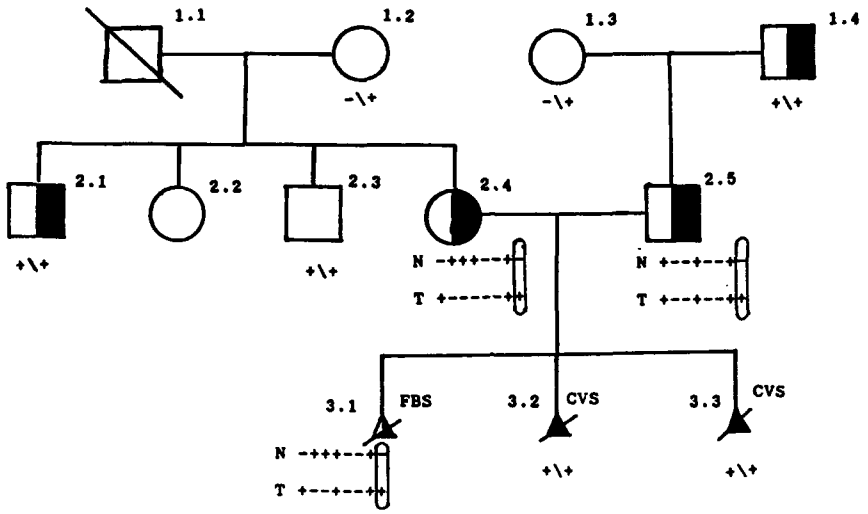


FIGURE 5. Linkage studies in a family where a misdiagnosis occurred. The haplotypes represent the β-globin gene polymorphic sites: 5′ *Hinc* II/ε, *Hind* III/$^{G}\gamma$, *Ava* II/ψβ, *Hinc* II/ψβ, *Ava* II/β, and *Hinf* I/β 3′. The most informative polymorphic site is *Hinf* I/β (*enclosed*). (+) polymorphic site present, (−) absent; (N) normal, (T) thalassemic haplotype. Pregnancy of fetus 3.1 was terminated after fetal blood sampling (FBS) and diagnosis of thalassemia major; linkage studies show that this fetus was a heterozygote. Pregnancies for fetuses 3.2 and 3.3 were terminated after CVS and DNA diagnosis. (□) Male, (○) female, (◨, ◑) heterozygotes, (■, ●) homozygotes: thalassemia major. *Triangles,* sex not determined.

pregnancy, we have felt it necessary to carry out extensive family studies in order to confirm the homozygote diagnosis in the fetus that was aborted. In two cases (TABLE 2) DNA studies showed that the original diagnoses were mistaken, and, fortunately, further misdiagnoses were avoided in these cases. Had the inaccurate fetal diagnosis

TABLE 3. Misdiagnoses in the DNA Diagnostic Service

Case	At Risk[a]	Diagnosis	Actual Result	Cause of Misdiagnosis
1.	α-Thal	α-Thal trait	Hydrops fetalis	Plasmid contamination
2.	β-Thal major	β-Thal trait	β-Thal major	Partial digestion
3.	HbSS	HbAS	HbAA	Maternal contamination
4.	HbSS	HbAS	HbS/β-thal	Misdiagnosis in parent
5.	HbSS	HbAS	HbSC	Misdiagnosis in parent
6.	β-Thal major	β-Thal trait	β-Thal major	Maternal contamination
7.[b]	β-Thal major	Normal	β-Thal trait	Wrong assignment of linkage[b]
8.[b]	β-Thal major	β-Thal trait	β-Thal major	Wrong assignment of linkage[b]

[a]Thal, thalassemia.

[b]Cases 7 and 8 were in the same family. A prior pregnancy was aborted after the fetus, at risk for β-thalassemia major, was diagnosed to have β-thalassemia major by the globin chain biosynthesis test. This fetus was later found to be unaffected. The fetal material was used for linkage studies. However, the DNA gene mapping studies resulted in an incorrect assignment of linkage, leading to the birth of an affected child in case 8.

TABLE 4. Maternal Contamination in CVS Samples

Center[a]	Total Studied[b]	Contaminated with Maternal Tissue (*n*)
UCH (London)	91	1
King's College	17	0
Other U.K.	25	0
Overseas	28	10
Total	161	11

[a]UCH, University College Hospital.
[b]Studies done with 3′ αHVR and pλg3.

been used as the basis for further DNA diagnosis, the couples would each have been involved in a series of errors. FIGURE 5 shows the linkage studies for one of these families.

Most CVS samples for DNA studies are sent to the national laboratory in Oxford for a diagnosis. In a total of 759 cases, there have been 8 misdiagnoses (TABLE 3). Two of these were due to misdiagnosis in a parent and two to maternal contamination. In one family there have been three misdiagnoses: the first by globin chain biosynthesis and two later ones by DNA gene mapping studies, leading to the birth of an affected child.

In a separate study, 161 DNA samples from chorionic villus tissue were studied with the 3′ αHVR and pλg3 probes[14,15] to determine the presence of any maternal tissue contamination (TABLE 4). Eleven out of 161 samples had some degree of maternal contamination (TABLE 5). This was not enough to cause misdiagnosis with the method used (Southern blotting) but could possibly cause problems with diagnosis based on gene amplification using the polymerase chain reaction and RFLPs or based on the amplification refractory mutation system (ARMS).[16,17] The study showed that more experienced centers have less likelihood of producing contaminated samples.

HOW TO OFFER SCREENING AND COUNSELING?

In the United Kingdom, at-risk couples are still most often identified in the antenatal clinic, but there are many disadvantages in waiting so late to start the process of testing and counseling. The option of whether to undertake a pregnancy at all is no longer open; at-risk couples are usually identified too late for first-trimester prenatal diagnosis; emotional involvement makes choice unnecessarily difficult and painful; some women present too late in pregnancy for prenatal diagnosis to be

TABLE 5. Results of 11 Samples Contaminated with Maternal Tissue

n	Result
4	Normal
5	Heterozygote
1	Fetal loss
1	Uninformative by linkage studies but 100% maternal

possible; there is no time to correct clinical or laboratory errors; the short deadline often results in a hurried decision; the level of awareness of the obstetrician is not always what it might be; and rapid turnover of antenatal staff makes it difficult to sustain a consistent policy. Clearly, screening and counseling for the hemoglobinopathies should be offered at the preconception stage, i.e., these services should be part of family planning and integrated into primary health care.[12] This is starting to happen, but a great deal of effort will have to be devoted to increasing public awareness and to professional education about genetic risks in general before screening and counseling become routine in primary care.

In conclusion:

1. A widely disseminated service can lead to an increased misdiagnosis rate: but misdiagnosis can be avoided if all at risk couples are referred to specialist counseling and prenatal diagnosis centers.
2. The service objective of providing an informed choice for every couple at risk for a hemoglobinopathy can only be achieved by developing appropriate screening and counseling strategies for each ethnic minority group.
3. It seems that the appropriate place to initiate the genetic screening services is in primary health care. Appropriate genetic educational training programs for primary care workers must be developed.

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Prenatal Diagnosis of Thalassemia in South China[a]

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INTRODUCTION

Both α- and β-thalassemias are common genetic disorders in South China. The gene frequency of each of these disorders is estimated to be 3–5% in the province of Guangdong, which has a total population of over 50 million. The gene frequencies in other provinces in South China are similar or even higher.

It is now possible to obtain cells of fetal origin either by amniocentesis or by chorionic villus biopsy. In addition, recent advances in molecular biology have simplified considerably the DNA diagnostic techniques for the thalassemias.[1] A prenatal diagnosis laboratory for thalassemias was established in the Nan Fang Hospital of the First Military Medical University in Guangzhou, the capital of Guangdong Province, in South China. This laboratory serves Guangdong, Guangxi, Fujian, and Hainan provinces. This paper reports the results of the diagnostic work carried out in this laboratory during 1989.

DIAGNOSIS OF α-THALASSEMIA

The α-thalassemia of clinical significance in South China is usually due to the deletion of the Southeast Asian type, involving all ψα- and α-globin genes on the short arm of chromosome 16. This deletion is commonly referred to as $--^{SEA}/$ and can be clearly demonstrated by Southern blot analysis.[2] In 1987, Chehab and colleagues[3] devised a polymerase chain reaction– (PCR) based method for the diagnosis of homozygous α-thalassemia, utilizing primers which would amplify 136 bp of DNA normally present between the ψα1- and α2-globin genes. This DNA

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segment is deleted in α-thalassemia of the $--^{SEA}/$ type. Concomitantly, a 110-bp DNA fragment of the β-globin gene is also amplified, which serves as the control for the PCR. In homozygous α-thalassemia, otherwise known as hemoglobin (Hb) Bart's hydrops fetalis syndrome, the 110-bp β-globin gene fragment is present after PCR, whereas the 136-bp α-globin gene fragment is absent.[3] This method, which is relatively simple and rapid, has been adopted by our laboratory as the diagnostic test. We have performed 38 prenatal diagnoses on pregnancies at risk for homozygous α-thalassemia, and 5 fetuses with homozygous α-thalassemia were identified.

DIAGNOSIS OF β-THALASSEMIA

There are now 15 known β-thalassemia point mutations in the Chinese population.[4] In order to identify the more commonly encountered mutations in South China, we have studied 236 chromosomes with β-thalassemia by the technique of allele-specific oligonucleotide hybridization to amplified fragments of the β-globin gene. The data indicate that four mutations, a 4-bp deletion in codon 41–42 (−CTTT), a C→T mutation in IVS-2 at position 654, an A→T nonsense mutation in codon 17 and an A→G mutation at TATA box position −28, account for over 90% of all the mutant alleles studied (TABLE 1).

For prenatal diagnosis of pregnancies at risk for homozygous or compound

TABLE 1. β-Thalassemia Mutations and Their Frequencies in South China

Mutation[a]	Frequency (%)
Codon 41–42 (− CTTT)	49.6
IVS-2 nt 654 (C→T)	18.8
Codon 17 (A→T)	15.8
TATA box nt −28 (A→G)	9.0
TATA box nt −30 (T→C)	1.5
Codon 71–72 (+A)	0.8

[a]Number of chromosomes = 236. nt, nucleotide.

heterozygous β-thalassemias, DNA was extracted from the peripheral blood cells of the couples under investigation. DNA was also extracted from chorionic villi obtained at 7–11 weeks of gestation or from amniotic fluid cells obtained at 16–20 weeks of gestation. *In vitro* amplification of DNA was performed as described using two sets of primers to amplify the β-globin gene regions.[5] Amplified DNA was dotted onto nylon filters, which were then hybridized with horseradish peroxidase– (HRP) labeled allele-specific oligonucleotide probes. Six pairs of probes were used, four corresponding to the four common β-thalassemia mutations found in the Chinese, as mentioned above, as well as pairs of probes for the frameshift mutation in codon 71–72 (+A) and the G→C mutation in IVS-1 at position 5. The presence of an oligonucleotide hybridized to amplified DNA is detected by histochemical staining for presence of the enzyme.[6]

Thirty-four cases of prenatal diagnoses of β-thalassemia were performed. The results are shown in TABLE 2. Nine fetuses inherited β-thalassemia alleles from both of their parents.

DIAGNOSIS OF β-THALASSEMIA BY DNA SEQUENCING

When the β-thalassemia mutation is not detected with oligonucleotide probes specific for the six common mutations, the entire β-globin gene can be amplified and sequenced directly to identify the mutation. During the course of prenatal diagnosis for β-thalassemia in a Fujian couple in 1988, we encountered a mutation that was not detectable by oligonucleotides for the Chinese mutations then known. Amplification of the β-globin gene and direct DNA sequencing revealed a previously undescribed T→C TATA box mutation, which was carried by the father but was not inherited by the fetus. Even in this complicated case, prenatal diagnosis was accomplished within two weeks.[7]

DISCUSSION

Carrier detection, genetic counseling, and prenatal diagnosis are indispensable components in the prevention of thalassemia major among populations in which the gene frequency for thalassemia is high, as it is in South China. The introduction of the PCR technique offers several advantages over previous prenatal diagnostic methods.[8-10] *In vitro* DNA amplification can be performed rapidly. The increased number of target sequences following amplification permits the use of less sensitive probes, i.e., ^{35}S-labeled oligonucleotides or non-radioactive probes such as the horseradish peroxidase–labeled oligonucleotides used in the present study. ^{32}P-labeled oligonucleotides, despite their short half-life, can also be used for up to two to three months. Thus, the PCR technique has significantly simplified the DNA diagnostic procedure. More recently, we have shown that denaturing gradient gel electrophoresis also offers another non-radioactive means of detecting multiple mutations for β-thalassemias and other genetic disorders.[11]

TABLE 2. Prenatal Diagnosis in 34 Fetuses at Risk of β-Thalassemia

Diagnosis	Mutations	*n*
Normal	—	5
Heterozygote (total)		20
	Codon 41–42	11
	IVS-2 nt 654	3
	Codon 17	4
	TATA box nt −28	2
Homozygote or Compound Heterozygote (total)		9
	Codon 41–42/IVS-2 nt 654	3
	Codon 41–42/codon 17	3
	Codon 41–42/codon 41–42	2
	Codon 41–42/TATA box nt −28	1

The success of implementing carrier detection and prenatal diagnosis in decreasing significantly the number of live births affected with thalassemia major has been amply demonstrated in a number of Mediterranean regions.[12] The present report clearly demonstrates that prenatal diagnosis for both α- and β-thalassemias can be successfully carried out in South China. With the appropriate funding and resources,

a more comprehensive program on screening, genetic counseling, and prenatal diagnosis can be established in South China. With these genetic services in place, it is anticipated that much progress can be made in the prevention of thalassemia major in South China.

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Current Therapy for Thalassemia in Italy

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The length and quality of life of thalassemic patients have been greatly improved in Italy during last few years. A high standard of treatment has been made equally available to all patients living in different regions of the country.

Many factors have played an important role in determining this improvement. The importance of the problem, in terms of high patient numbers (more than 5000), gave rise to social and scientific interest on the part of some organizations. They started promoting awareness at many levels in cooperation with the World Health Organization (WHO) Working Group on Hereditary Anemias and the National Association for Pediatric Oncology and Hematology (AIEOP).

Since adequate treatment of thalassemia depends not only on a high level of technology but also on the regular application of a therapeutic scheme and on the meticulous analysis and monitoring of clinical data, two instruments have been crucial in improving treatment quality: the national protocol and the standardized clinical record.

The management guide for transfusion-dependent patients has been worked on since 1980.[1] Distributed by the AIEOP and the parents' and patients' associations, it is widely accepted and regularly updated for new results and problems arising. It has been sponsored by WHO.[2]

A standardized format for recording clinical data was proposed in 1982.[3] During the last few years a computerized clinical record (Computhal) was drawn up in Italian and English and distributed to many clinical centers.[4] Computhal is organized on three levels: the first for regular patient follow-up, the second for the study of specific clinical problems, and the third for statistical analysis. The calculation of transfusional indices[5] and the identification of some other key indicators of the patient's clinical status, such as ferritin levels, compliance and growth in height, made it possible to pool and compare results in different centers and paved the way for the organization of cooperative trials.

Two other ventures were started to ensure prevention and quality control of treatment.

A national registry for transfusion-dependent patients was set up and coordinated by the AIEOP. The registry, updated every three years, allowed the evaluation of the number of patients and their age distribution, the identification of higher prevalence areas, and the calculation of birth and death rates, both at regional and national levels.[6]

Cooley Care is a cooperative quality assurance program proposed in 1984, which initially aimed at verifying the transfusional indices and the quality of care both nationally and internationally.[7] A central computerized data base was created, and in 1988 the program was extended to evaluating specific problems such as iron chelation, immunization, human immunodeficiency virus (HIV) infection, and the genetic aspects of thalassemia.

One of the most interesting and useful results of these cooperations was the spontaneous aggregation of many groups into trials and research projects on basic

and clinical problems. Multicenter clinical trials provided information on many pathological and therapeutic aspects of the disease. The prevalence and the variation and severity of different complications, such as liver involvement,[8] growth retardation and failure of puberty,[9] and diabetes,[10] as well as the causes of death,[11] were studied in a large patient population.

Furthermore, pilot studies were carried out in some centers on the molecular defects[12-15] and on problems arising, such as therapy for some endocrine disorders,[16,17] desferrioxamine pharmacokinetics and toxicity,[18-22] and intensive chelation.

Great attention was paid to psychological and social aspects.[23-25] An international program aimed at studying psychological problems and trying out methods for the best psychological support was promoted.

In this paper attention will be focused only on some results obtained from the cooperative studies or by single groups determining modifications of the treatment protocol. These results apply to transfusion schemes, to chelation therapy, and to desferrioxamine toxicity.

For transfusion-dependent patients, a high transfusion scheme, in which the mean hemoglobin level is held around normal levels, is recommended. It has been demonstrated that, by shifting from a very low to a high transfusion protocol, a reduction of bone marrow expansion and of blood volume is obtained, owing to a decrease of the quantity of blood necessary for maintaining a given hemoglobin level.[26,27] From these results the treatment guide recommended a pre-transfusion hemoglobin level of 10.5–11 g/dl. On the other hand, patients regularly transfused with a protocol adequate for inhibiting bone marrow activity maintain a constant blood volume. In this situation a correlation has been demonstrated between the mean hemoglobin level maintained and the blood transfusion requirement in 174 splenectomized patients followed in the centers at Torino, Milano, and Ferrara (FIG. 1). These data fit in with that of Modell and Berdoukas[5] and with the Cooley Care Program results.[7] It has also been said that the blood requirement varies with the transfusion interval.

Recently in our center we have designed a randomized trial in order to measure the effect of allowing a reduction in the mean hemoglobin level (from 13 to 12 g/dl) in splenectomized and unsplenectomized patients, and of lengthening or shortening the transfusion interval (16 days versus 34 days).[28] The results are illustrated in TABLE 1.

The blood requirement fell in all patients with a lower mean hemoglobin level, and no difference in blood consumption was found between the two groups with different transfusion intervals. Therefore, the most important parameter influencing blood requirements in regularly transfused patients is the level at which mean hemoglobin is maintained. We have not seen a clinically demonstrable blood expansion in patients subsequent to the reduction of the mean hemoglobin level from 13 to 12 g/dl or in patients kept at a lower pre-transfusion hemoglobin (9.5 g/dl). On the basis of these results, a pre-transfusion hemoglobin of 9.5–10 g/dl and a mean hemoglobin level of 12 g/dl are recommended in the updated guide for management.

Intensive chelating therapy by continuous subcutaneous infusion of desferrioxamine (DFO) has been used systematically in Italy since 1978. Its effectiveness as a means of preventing or reducing the iron overload and of prolonging patient survival has been widely demonstrated. It is, however, impossible to constantly maintain negative iron balance in all patients, due to the difficulties of achieving high compliance and due to the appearance of toxic effects of DFO which necessitate a reduction in the chelation intensity.

It is necessary to deal with the compliance problem by keeping in mind that not only does chelation present some practical difficulties but, above all, many psycholog-

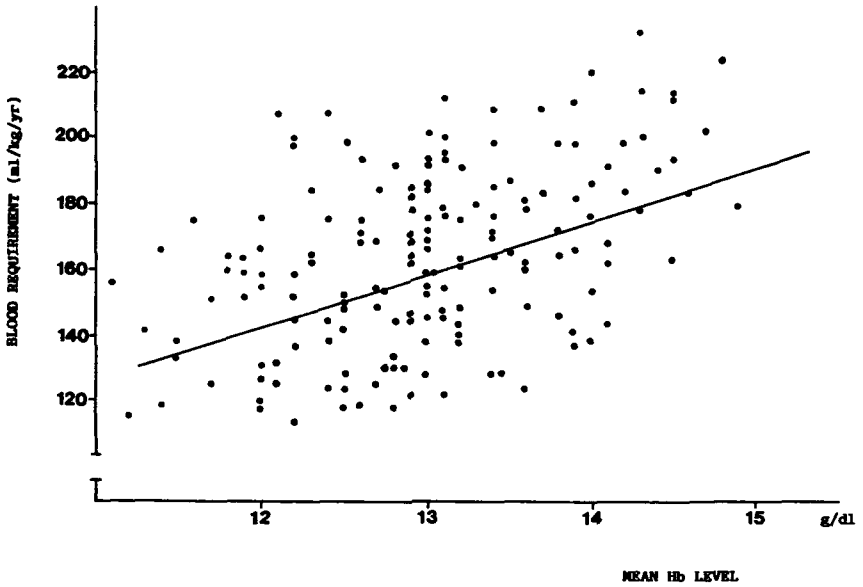

FIGURE 1. Correlation between mean hemoglobin (Hb) level maintained and blood requirement in 174 splenectomized patients.

ical problems. Numerous efforts have been made in the past to give psychological support to patients and their families.[24,25]

FIGURE 2 illustrates the mean ferritin levels observed during 1989 in 384 patients of different ages treated in the Torino and Ferrara centers. The data were obtained through the Computhal program. Most of the patients, irrespective of age, have ferritin levels maintained between 1000 and 2500 mg/dl. These results indicate that iron overload can be kept within a probably safe range in young patients and can be drastically reduced in old, heavily overloaded subjects. It is also possible to deduce that compliance can be improved at every age.

The trend of the mean ferritin level in our patients, calculated every year from 1979 to date, is illustrated in FIGURE 3. The ferritin levels of the whole population fell slowly, and after 1985 we observed a slight increase. This increase was the consequence of reducing the intensity of chelation, a change we had decided to adopt

TABLE 1. Effect of Different Transfusion Protocols and Hemoglobin Levels on the Blood Requirement in 50 Splenectomized Patients

Protocol	Hb (g/dl)[a] Pre	Post	Mean	Blood Requirement (ml PRC/kg/yr)[b]	Transfusion Interval (days)
1986	11.0 ± 0.3	14.9 ± 0.4	13.0 ± 0.3	163.7 ± 37	25.2 ± 2.0
1987	10.9 ± 0.3	14.7 ± 0.5	12.8 ± 0.3	163.0 ± 27	26.6 ± 2.5
1988A	9.7 ± 0.5	14.2 ± 0.6	11.9 ± 0.2	147.8 ± 24	16.0 ± 1.3
1988B	11.0 ± 0.3	13.4 ± 0.4	12.2 ± 0.1	149.7 ± 18	31.3 ± 3.4

[a]Pre, pre-transfusion; Post, post-transfusion.
[b]PRC, packed red cells.

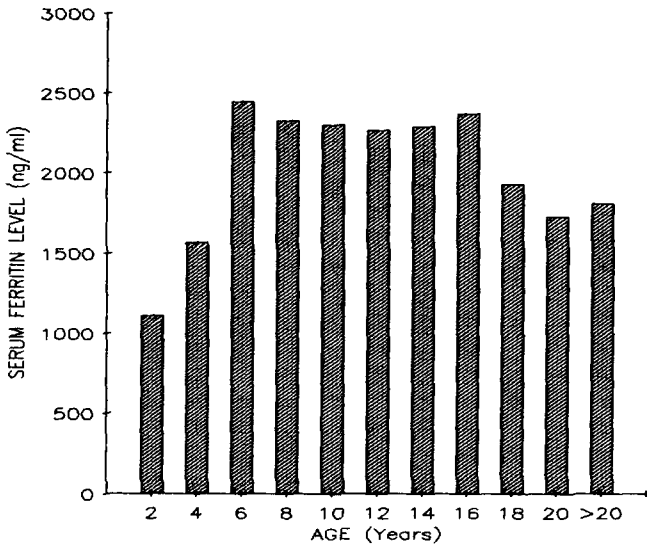

FIGURE 2. Mean ferritin levels observed in 1989 in 384 patients of different ages.

after the appearance of toxic effects of DFO. In fact, serious complications of chelation treatment on growth[18,19] and on visual and auditory functions[20,29–31] have recently been described.

During 1985 growth velocity was impaired in more than 50% of our prepuberal

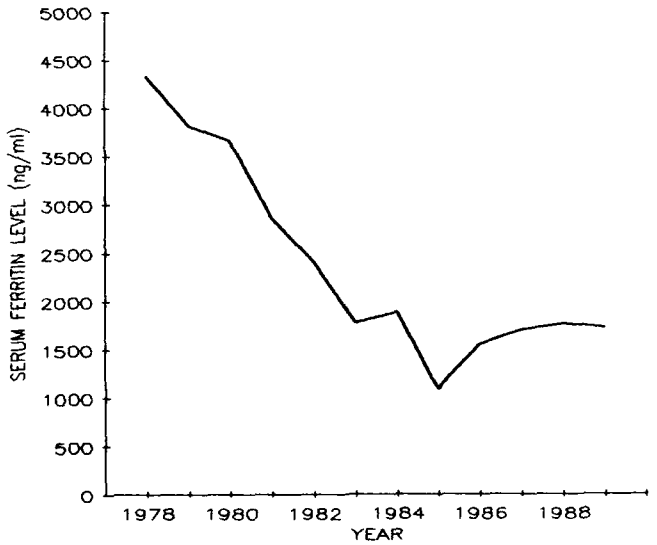

FIGURE 3. Mean ferritin levels observed from 1979 to 1989 in patients at the Torino center.

patients. The defect was more frequent in subjects with low ferritin levels treated with high DFO dosages. Growth velocity resumed after reduction of the DFO administration schedule.[19] Since 1986, using a DFO dosage below 50 mg/kg, we have not observed impairment of growth in height in new cases.

Hearing loss, characterized by a high-frequency sensorineural defect, was observed in 38% of subjects. It appears to be correlated with DFO dosages and is more frequent in subjects with low ferritin l is.

The presence of auditory damage has been confirmed in an extensive cooperative study on 335 cases from seven different Italian centers.[32] A significant sensorineural hearing loss at over 50 dB was observed in 6% of the cases and a mild defect (threshold between 30 and 50 dB) in 21%. A control was carried out by re-examining all the 335 patients one year after the DFO dosage was reduced. Hearing function significantly improved among patients with a mild defect, while it only slightly ameliorated among those more severely affected.

It must be stressed that, in spite of these toxic effects, which can be largely prevented or reversed, chelation therapy has greatly improved survival by reducing the number of complications due to iron damage. In a recent study we observed that about 60% of well-chelated patients were free of serious complications at 20 years of age. On the contrary, less than 20% of poorly compliant patients of that age were living without complications.[33]

These data have been confirmed in a cooperative study on 1087 patients observed in seven Italian teaching hospitals.[11] Survival significantly improved in patients born after 1970 and treated with regular transfusion and subcutaneous chelation. This improvement was mainly due to a decrease in mortality from cardiac disease, and it has been demonstrated that cardiac disease can be prevented by intensive chelation.[34]

The possibility of improving the prognosis in thalassemia by updated management requires a high level of responsibility in all persons involved in providing health services, and close cooperation at basic and clinical research levels is essential.

SUMMARY

Care and life quality of thalassemic patients in Italy have greatly improved over the last years thanks to cooperation between many clinical centers. The achievement of the following points played an important role:

1. A national treatment protocol was adopted in 1980; it has been widely accepted and regularly updated.
2. A standardized format for recording clinical data and a computerized clinical record (Computhal) were adopted.
3. A national registry was set up in order to evaluate the patient age distribution and birth and death rates both at the regional and national level.
4. A quality assurance program (Cooley Care) was devised after key indicators for evaluating treatment were identified and a central data base was set up.
5. Cooperative clinical trials provided information on many pathological and therapeutical aspects of the disease (incidence of complications, causes of death).
6. Pilot studies were carried out on emerging problems (intensive chelation, desferrioxamine (DFO) pharmacokinetics and toxicity).
7. Attention was paid to psychological and social problems.

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Current Therapy of Cooley's Anemia

A Decade of Experience with Subcutaneous Desferrioxamine[a]

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INTRODUCTION

Many advances in the management of thalassemia major (Cooley's anemia) have developed over the past several decades. Their impact has been realized with the efficacious use of transfusions and splenectomy and the development of the subcutaneous use of desferrioxamine (s.c. DFO).

Current management programs prevent excessive iron accumulation and promote enhanced iron excretion. The goal of management is to achieve optimal iron balance. Excessive iron accumulation is thwarted by the timely use of blood transfusions and the careful monitoring of annual transfusion requirements to identify early hypersplenism in order to perform a splenectomy at the appropriate time. Iron excretion is enhanced with the use of prolonged infusions of the iron chelator desferrioxamine.

Iron balance should be individualized to meet the specific needs of the patient. To achieve optimal iron balance, one must consider the lifetime transfusional iron burden as well as the current transfusional iron load, the age, the degree of hypersplenism, various serum iron parameters, and evidence of parenchymal iron damage. The optimal balance for an individual may be 100% balance, i.e., the current excretion of iron equal to the current transfusional iron load, or negative iron balance, i.e., the current excretion of iron exceeding the current transfusional iron load.

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Our understanding of the use of subcutaneous (s.c.) and intravenous (i.v.) DFO and its efficacy and toxicity have been expanded over the past decade. Dose-response curves have been defined, and we are aware that both urinary and fecal iron excretion should be considered in order to achieve optimal iron balance and avoid toxicity. DFO efficacy can be increased with splenectomy as well as with dose escalation. High-dose i.v. DFO can rapidly remove large amounts of iron and lower serum ferritin levels. However, it is still unclear when is the ideal time to initiate s.c. DFO in the newly diagnosed, minimially iron-loaded patient and what are the full benefits of high-dose i.v. DFO for the symptomatic patient with severe hemochromatosis.

This discussion will address our current recommendations regarding transfusion therapy, the indication for splenectomy, and the s.c. DFO dose regimens required to promote enhanced iron excretion and achieve optimal iron balance as, well as our clinical experience with these approaches over the past decade.

TRANSFUSION PROGRAM

Our current transfusion program has benefited from the historical contributions of many investigators. Originally, red blood cell transfusions were administered to sustain life and relieve anemic symptoms. However, adherence to the 1964 recommendation of Wolman to maintain a higher baseline hemoglobin level afforded an improved state of health.[1] Since the 1960s, "hypertransfusion" regimens, wherein hemoglobin (Hb) levels are maintained above 10 g/dl with a mean of 12 g/dl from infancy have prevented bony malformations and cardiomegaly and have allowed for improved growth and development in the first decade.[2,3] The expanded blood volume associated with a profound anemia is reduced with hypertransfusion; however, in 1980, Propper *et al.* advocated the use of a "supertransfusion" regimen designed to maintain a mean Hb of 14 to 15 g/dl, thereby further suppressing endogenous erythropoiesis, reducing bone marrow mass and blood volume, while simultaneously reducing plasma iron clearance and tissue iron deposition.[4] Whether it is clinically of greater benefit in the long term to maintain supertransfusion therapy in contrast to a hypertransfusion program has yet to be determined. However, maintaining a higher Hb level of about 10 g/dl tends to reduce the hyperabsorption of dietary iron.[5] In 1980, Piomelli reported that once the baseline Hb is raised above 10 g/dl, transfusion requirements increase by as much as 20% for maintenance of a single gram increment in the Hb level.[6] In addition, the narrowing of the interval to three weeks or less between transfusions would afford a 20% or more reduction in yearly iron load.[7]

Historically, we have maintained a transfusion interval of two weeks in order to avoid raising the post-transfusion Hb level above normal and to maintain a more normal physiologic Hb range pre- and post-transfusion.

We transfuse 6–8 ml of packed red blood cells (pRBC) per kilogram of body weight per visit. We maintain a pretransfusion Hb level of 11.0 ± 0.5 g/dl. However, we do not split a donor unit; and, on the basis of body weight, we will alternate the number of units of blood required per visit and/or have additional weekly visits to meet the yearly requirement of approximately less than 200 ml/kg per year. We never exceed the transfusion of 2 units of pRBC in one outpatient clinic visit. We transfuse each pRBC unit over 1.5–2 h, unless the individual has cardiovascular compromise, in which case we will diurese and transfuse a unit of pRBC over 3 h.

Various transfusion programs select the type of blood product transfused on the basis of availability, cost, and patient sensitization. Urticarial and febrile transfusion

reactions commonly occur and can be reduced or avoided with the use of leukocyte-poor and washed pRBC. Currently, leukocyte-poor and washed red cells can be prepared with several techniques. Red blood cells which are frozen with glycerol, thawed, and subsequently washed with saline (FTpRBC) were previously the only leukocyte-poor preparation available. Recently, it has become possible to reduce leukocyte numbers with the use of cotton wool filters and/or Pall filters; however, washing of these cells with saline is still required to efficaciously remove excess plasma proteins.[8,9]

Since the early 1970s, we have administered frozen-thawed and washed pRBCs to prevent the unpleasant febrile and urticarial transfusion reactions and to avoid the leukocyte sensitization which may otherwise occur.

SPLENECTOMY PROGRAM

Hypersplenism, which results in the excessive splenic destruction of red blood cells, is an inevitable consequence of chronic transfusion therapy. It usually develops by 6 to 8 years of age, as a progressive increase in yearly transfusion requirements. We recommend splenectomy when the yearly transfusion requirement exceeds 250 ml/kg per year.[10] In addition, regardless of the age at splenectomy or the interval since splenectomy, a pre-transfusion Hb greater than 10.5 g/dl with a yearly transfusion requirement of less than 200 ml/kg per year can be maintained. Since the ability to achieve iron balance on DFO is a function of transfusion requirement as well as dose, reducing the transfusion requirement by splenectomy affords better iron balance and can minimize the dose of DFO required to achieve balance.[10]

Overwhelming sepsis with pneumococcus and other enteropathogens may occur in the splenectomized thalassemic.[11] In addition, altered immunity, as reflected in altered lymphocyte subset populations and decreased cytotoxicity, occurs as the number of pRBC units transfused increases.[12]

We recommend, prior to splenectomy, vaccination against pneumococcus and hemophilus influenza. Postsplenectomy, antimicrobial prophylaxis is instituted, usually penicillin at 250 mg twice daily, or another agent if there is allergy to penicillin.

We maintain an aggressive approach to infection control after splenectomy. In addition to antimicrobial prophylaxis, the febrile patient with fever greater than 38.5°C presenting with no focal signs of infection is presumed septic and placed on broad-spectrum intravenous antibiotics until blood, urine and throat cultures demonstrate no microbial growth after 72 h of incubation, at which time appropriate therapy is then selected.

CHELATION PROGRAM

Despite management programs to reduce transfusional iron loading, a cumulative iron burden is an inevitable function of the number of lifetime transfusions and, hence, age. Since the body has no physiologic mechanism to excrete excess iron, an iron chelator must be used. Desferrioxamine (Desferal) is currently the only FDA-approved chelator available for clinical use. It is produced from *Streptomyces philosis* and has high affinity for iron (binding constant equal to 10^{31}). It is readily excreted in urine and stool; but, unfortunately, it has a short half-life of 76 min and must be administered parenterally.[13]

In the mid 1970s, we and others demonstrated that the effectiveness of DFO

could be greatly enhanced by continuous administration and that subcutaneous infusions are 80% as effective as an equivalent intravenous dose over a 24-h period.[14] The advent of the small, portable, battery-operated syringe pump allowed prolonged infusions of s.c. DFO administered over 8–12 h a day to become standard therapy.[15]

The effectiveness of desferrioxamine depends upon the lifetime iron burden, the available iron pool, the rate at which the pool is repleted, and the dose. Previously, we reported that the i.v. DFO dose required for a particular urinary iron excretion response is a function of age, or the transfusional lifetime iron burden. We demonstrated that increasing the dose of i.v. DFO in increments from 20 to 40, 60, and 80 mg/kg gave little increase in urinary iron excretion in younger patients (between 5 and 10 years of age). In contrast, it induced a linear increase in older patients (greater than 16 years); however, in those 10 to 15 years of age excretion rose to a plateau at 40 to 60 mg/kg.[14]

In 1982, Pippard *et al.* measured both urinary and stool iron excretion and similarly showed that, as the i.v. dose of DFO increases, urinary iron excretion reaches a plateau but stool iron excretion continues to rise and may account for up to 70% of excretion with DFO doses up to 150 mg/kg per day.[16] Therefore, stool iron excretion plays a considerable role in excretion at escalated DFO doses.

Since DFO induces iron excretion in both urine and stool, and the goal of chelation therapy is to achieve iron balance or even net negative iron balance, the contribution of stool as well as urinary iron balance should be considered when calculating iron balance.

We have measured "total" iron balance, i.e., balance from both urinary and stool iron excretion, in a group of heavily iron-loaded thalassemic patients aged 10 to 28 years. Metabolic iron balance studies were performed for each patient in our clinical research unit. Over a 7-day period, each patient received a daily dose of s.c. DFO, either 20, 40 or 60 mg/kg over an 8-h infusion, while on a standardized low-iron diet. Daily 24-h collections of urine, stool, and uneaten food were measured for iron content by atomic absorption spectroscopy (AAS). Total balance was determined for each patient by averaging the amount of daily urine and stool iron excretion and multiplying by 365 days, assuming daily compliance with yearly use of DFO. Yearly transfusional iron load was calculated from the number of pRBC units transfused per year, multiplied by 167 mg (the amount of elemental iron in one unit FTpRBC as measured by AAS).

Urinary iron balance, representing the contribution of urinary iron excretion, increases with each escalation of the DFO dosage but reaches a plateau at 40 to 60 mg/kg. However, stool iron balance, representing stool iron excretion, increases in a more linear fashion. Stool iron balance approximates 60% of the total iron balance (133%/228%) at 60 mg/kg, 50% (97%/200%) at 40 mg/kg, and 30% (54%/153%) at 20 mg/kg of daily s.c. DFO. Although *net* negative iron balance (53% ± 21%) is achieved in these heavily iron-loaded patients at 20 mg/kg, a greater net negative balance of over 100% is achieved at 40 mg/kg (100% ± 13) and at 60 mg/kg (128% ± 24) of s.c. DFO. (FIG. 1).

Total iron balance studies in response to varying doses of s.c. DFO were evaluated in terms of patients' ages, for five groups of patients: those at <5 years of age, at ≥ 5 to 10 years, at ≥ 10 to 15 years, at ≥ 15 to 20 years, and at ≥ 20 years. We observed that at 20 mg/kg of s.c. DFO, only the older patients, those at or greater than 15 years of age, could achieve a net negative balance greater than 50%. Increasing the dose to 40 mg/kg allowed patients at or over the age of 10 years to achieve a net negative balance greater than 50% and those less than 10 years to achieve a balance. Escalating the dose to 60 mg/kg enabled all patients, even the youngest, less than 5 years of age, to achieve 50% net negative balance (FIG. 2).

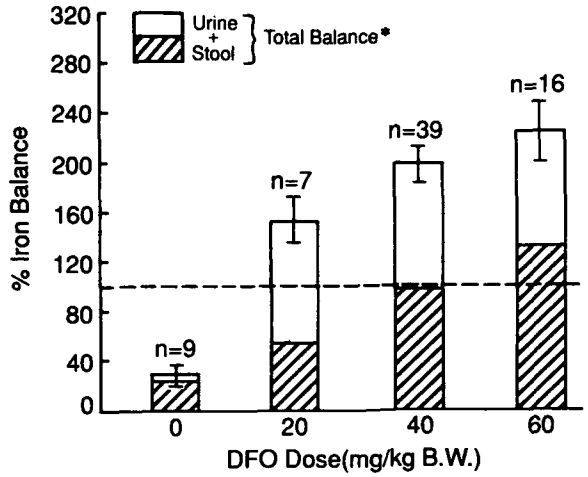

FIGURE 1. Iron balance attained in response to varying dosages of s.c. DFO demonstrates that urinary iron balance increases but reaches a plateau with escalated dosage, whereas stool iron balance increases in a more linear fashion. Stool iron balance approximates 60% of total iron balance at 60 mg/kg of daily s.c. DFO/kg of body weight, 50% at 40 mg/kg, and 30% at 20 mg/kg.

The optimal time to initiate chelation therapy in the very young and newly diagnosed child is still under investigation. It is apparent that age, iron load, and the dose-response pattern must be carefully considered. In 1988, De Virgiliis *et al.* reported that initiating chelation concomitantly with transfusion therapy in infancy, with serum ferritin levels less than 1000 ng/ml and with a mean DFO dose of 70

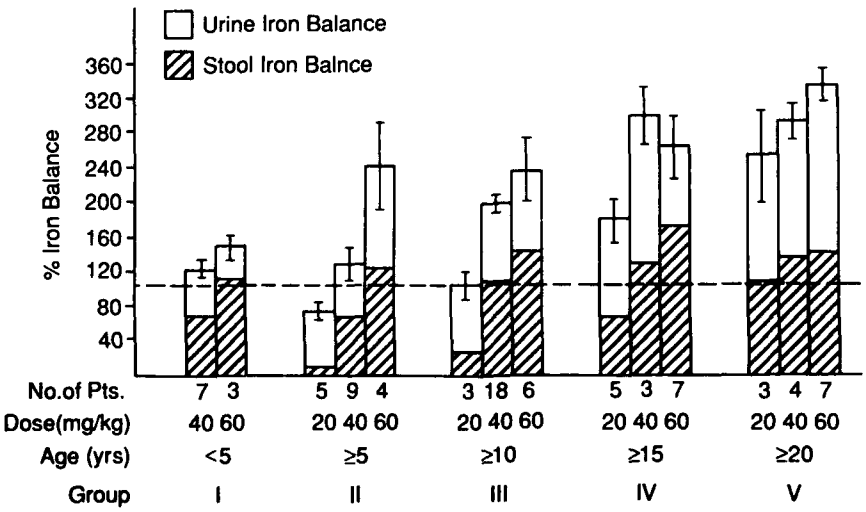

FIGURE 2. The total iron balance dose-response pattern at 20, 40, and 60 mg/kg of s.c. DFO over a daily 8-h infusion varies with the patient's age and/or iron burden.

mg/kg per day, may contribute to growth retardation and radiographic epiphyseal plate disturbances which are seen less frequently in patients begun on similar doses at 3 years of age.[17]

In a group of our complaint patients begun on DFO therapy at 5 to 10 years of age and maintained on escalating doses of s.c. DFO (20, 40, and 60 mg/kg per day) to achieve total iron balance until approximately 10 years of age and net negative balance thereafter, we observed more normal height-growth attainment.[18] However, two of five patients treated with DFO at 40 and 60 mg/kg per day at less than 3 to 4 years of age, with serum ferritin levels greater than 1000 ng/ml, developed growth disturbances and radiographic bone dysplasia.[19]

Desferrioxamine toxicity has been variably reported in several centers. Local irritation at the site of s.c. infusion associated with erythema and pruritis is commonly reported as a side effect. Neurotoxicity, including vision and hearing impairment, appears to occur more often in well-chelated younger patients or those receiving extremely high doses. Reduced visual-evoked potentials, cataracts, impaired color vision, and poor dark adaptation have been described, as well as a high frequency sensorineural hearing loss.[20] Fortunately, clinical improvement of visual and auditory side effects have been reported to be associated with dose reduction or temporary cessation of chelation therapy. The mechanism underlying the toxicity remains obscure. Disproportionately high DFO doses in relation to available body iron may result in high levels of unchelated drug. It is postulated that the unchelated drug may be directly neurotoxic or may deplete or chelate other trace elements when iron is not readily available.

High doses of ascorbic acid (vitamin C at 500–1000 mg/day) have also been reported to precipitate cardiac deterioration in heavily iron-loaded patients, possibly related to the mobilization of iron from nontoxic stores or the enhanced lipid peroxidation by iron in myocardial cells.[21] Since ascorbate deficiency associated with low leukocyte and serum levels occurs in thalassemia, we recommend the daily use of low-dose vitamin C, adequate to replete normal ascorbate levels, but not to exceed 2 mg/kg per day.

Currently, we recommend that s.c. DFO be administered over an 8-h infusion period, a minimum of 5 days per week. It should be initiated at least by 4 to 5 years of age in regularly transfused patients with serum ferritin levels greater than 1000 ng/ml. The appropriate s.c. dose, 20, 40 or 60 mg/kg, should ideally be selected on the basis of total iron balance based on both urinary and stool iron excretion. The young patient, between 5 and 10 years of age, should receive a dose (20 to 40 mg/kg per day) to achieve total iron balance and not to exceed 50% net negative total iron balance. The older unchelated or previously noncompliant patient who has a large iron burden should achieve net negative iron balance greater than 50% and as much as 100–200%, which can be achieved at s.c. DFO doses of 40–60 mg/kg per day. Optimally, dose regimens should be based on the individual dose-response pattern and iron load. These regimens should be monitored periodically, yearly if possible, and modified accordingly.

Clinical investigations are currently ongoing to evaluate the efficacy of high-dose i.v. DFO chelation to retrieve seriously compromised individuals, as well as those poorly compliant with the s.c. route of administration.[22] Further studies are also required to determine the optimal age and iron load at which to initiate iron chelation in the very young child.

CLINICAL PROGRESS WITH SUBCUTANEOUS THERAPY

Since we began our hypertransfusion and chelation program in the mid 1970s, we have annually monitored the clinical progress of our thalassemic patients. For nearly

12 years, we have regularly followed 68 patients who were begun on s.c. DFO between the ages of 5 and 28 years; an additional eight patients who began chelation at less than five years of age have been followed for close to five years. In accordance with our study protocol, the original patients were begun in 1977 on a daily DFO dose of 20 mg/kg, which was escalated over time to 40 mg/kg in 1982 and to 60 mg/kg in 1985. The eight youngest patients were begun on 40 to 60 mg/kg.

At entry into the chelation program and at the most recent point of observation, patients were evaluated in our clinical research center and monitored for iron, hepatic, endocrine, and cardiac status and s.c. DFO dose-response pattern. Iron status was determined by documenting the total lifetime number of units of pRBC transfused and calculated for lifetime transfusional grams of iron per killogram of body weight; the mean value of quarterly serum ferritin levels was also obtained. Hepatic status was evaluated by liver size and quarterly determinations of serum glutamic-oxalate transaminase (SGOT) and glutamic-pyruvate transaminase (SGPT) levels; liver biopsies were performed only at entry into the study for all but those less than five years of age. Hepatomegaly on physical examination was scored on the basis of centimeter size (S) measured beneath the midright costal margin. Liver biopsy results were scored from one to four points for the progressive degree of fibrosis and the presence of cirrhosis. Endocrine status was evaluated for height-growth by the number of standard deviations (SDS) of height attainment for chronological age (CA) against the normal mean for age and sex (HT-SDS-CA). A glucose tolerance test and screening for thyroid function as measured by T_3, T_4, and TSH levels were performed. Cardiac evaluations included an electrocardiogram (ECG), an echocardiogram, and a 24-h continuous ECG monitor. Baseline and annual ophthalmic and auditory examinations were also performed.

During the period of observation, patient compliance with the daily s.c. DFO dose and the frequency of use were addressed with a yearly written questionnaire completed by the patient and/or parent and by a quarterly verbal interview. The yearly cumulative dose of DFO in grams was calculated annually. A ratio of the total lifetime transfusional iron load in milligrams of iron at the start of the chelation to the cumulative DFO dose in grams at the time of observation was determined for each patient (DFO ratio).[23]

All patients were assigned to a group according to their age at the start of s.c. DFO therapy. Group I includes all those patients who were less than 5 years of age at the start of s.c. DFO, Group II patients were ≥ 5 years of age, Group III patients were ≥ 10 years of age, Group IV patients were ≥ 15 years old, and Group V patients were ≥ 20 years of age.

The condition of the patient groups at several time points will be considered—at entry into the chelation program, before DFO treatment, and after treatment with s.c. DFO at the latest time of observation, or at death—by comparing historically untreated patient groups with the age-matched treated patient groups.

Groups II, III, IV, and V were treated with s.c. DFO and observed over a mean of 10.6 years, whereas Group I was treated for 4.1 years. Group I, having attained a mean age of 6.3 years while on s.c. DFO, is compared to the similarly aged and untreated Group II at entry into the study. Similarly aged untreated patients at entry in Group IV at 15.9 years are compared to the similarly aged but treated Group II who attained the age of 15.7 years after 10.4 years of s.c. DFO therapy.

After 4 years of s.c. DFO therapy, those patients who attained the age of 6.3 years have better mean serum ferritin, SGOT, and SGPT levels and mean liver size in comparison to similarly aged 6.5-year-old untreated patients, despite similar lifetime iron loads (FIG. 3).

After 10.5 years of s.c. DFO therapy, those patients who attained the age of 15.1 years also have better mean serum ferritin and SGOT levels and mean liver size in

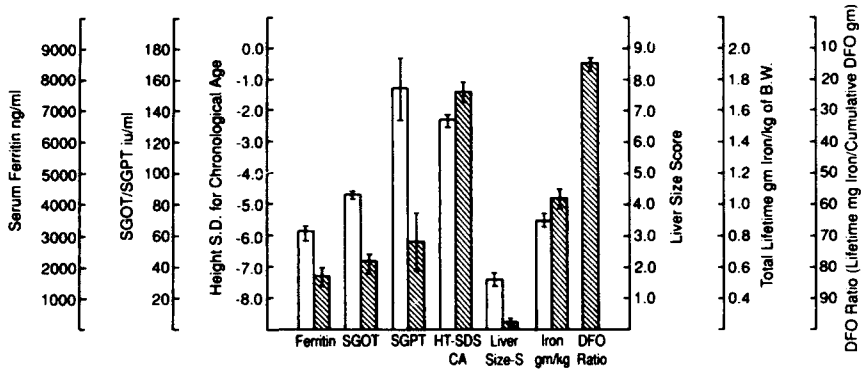

FIGURE 3. Nine patients treated for 4.1 years with s.c. DFO (*hatched bars*) who attained the age of 6.3 years have improved serum ferritin, SGOT and SGPT levels, HT-SDS-CA, and liver size (S) in comparison to 24 similarly aged historically untreated patients (*open bars*).

comparison to similarly aged 15.9-year-old untreated patients who had similar lifetime iron loads. (FIG. 4).

After 6 years of treatment, seven of the 14 patients who all began chelation at the mean age of 12.6 years have succumbed, at a mean age of 18.6 years. The surviving patients at the same age as those who succumbed had lower serum ferritin and liver biopsy scores, as well as better height-growth attainment. The DFO ratio was poorer in those who succumbed, despite similar treatment periods, suggesting less compliant DFO use.

Thus, it appears that significant factors for clinical well-being and survival involve the interplay of lifetime iron load, age, and liver disease at the start of therapy.

We have also addressed the clinical progress and survival of those patients with regard to their compliance with the regimen of DFO therapy and with regard to the frequency and cumulative amount of s.c. DFO administered relative to their pretransfusional iron burden at the time of initiation of chelation therapy, i.e. the DFO ratio.

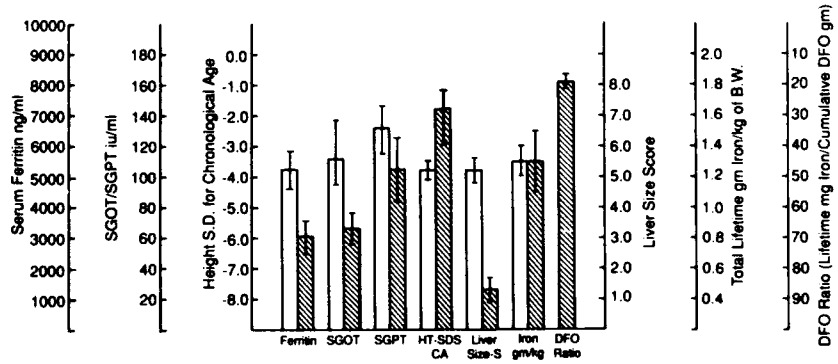

FIGURE 4. Twenty-four patients treated for 10 years with s.c. DFO (*hatched bars*) who attained the age of 15.7 years have improved serum ferritin and SGOT levels, HT-SDS-CA, and liver size in comparison to 13 similarly aged historically untreated patients (*open bars*).

To assess the role of s.c. DFO chelation to disease progression and survival, we evaluated the DFO ratios of patients at their latest observation point or at death in comparison to their transfusional iron burden at the start of chelation therapy. Clinical congestive heart failure resulting in death occurred in 24 patients with iron burdens > 0.74 g Fe/kg and DFO ratios ≥ 34.[24]

Endocrine disorders, including diabetes mellitus, hypothyroidism, and growth failure, are well-recognized complications of hemochromatosis. Diabetes developed in eight patients, hypothyroidism was identified in 19 patients, and height-growth deficits, defined as the number of standard deviations from the normal mean for height, were observed in 22 patients who began s.c. DFO with iron burdens ≥ 0.39 g/kg and DFO ratios of ≥ 24.

Patient survival (FIG. 5) was analyzed using the Kaplan-Meier product-limit method, and survival curves were compared using the log-rank test. All patients treated by the clinical service at the New York Hospital–Cornell Medical Center since 1960 were identified; birth date, date of death, or last day of follow-up if alive were ascertained by review of the hospital medical record. The patients were assigned to two groups based on their treatment regimens. Patients who went on to receive BMT (bone marrow transplant) and/or who succumbed with human immunodeficiency virus exposure or have been hypertransfused since diagnosis were excluded in this analysis. Group I consisted of 71 patients, with 52 deaths, who were treated with a low-transfusion regimen to maintain a pre-transfusion hemoglobin level of 7 to 8 g/dl and who received no DFO therapy. Group II consisted of 80 patients, with 30 deaths, who historically received a low-transfusion program which was increased to a hypertransfusion program in the 1970s and who were all begun on s.c. DFO therapy in 1977. The latter have an improved median survival of 31.0 years (mean 27.7 ± 0.7) compared to 17.5 years (mean 18.1 ± 0.7) in the low-transfusion group not treated with DFO.[23]

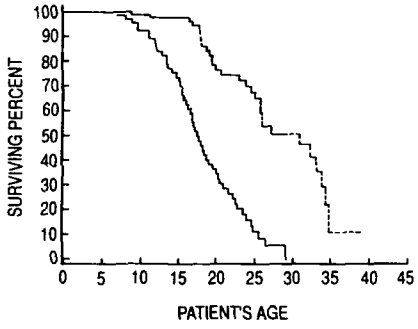

FIGURE 5. Patient survival using the Kaplan-Meier product-limit method and log-rank test. The *solid line* represents those patients treated on a low-transfusion regimen and with no chelation, who had a median survival of 17.5 years (mean, 18.1 ± 0.7 years), in comparison to those patients initially treated with a low-transfusion program, increased to a hypertransfusion program in the 1970s, and begun on s.c. DFO chelation in 1977, who have a median survival of 31.0 years (mean, 27.7 ± 1.0 years).

CONCLUSIONS

Our improved understanding of transfusion therapy and the role of splenectomy allows for the prevention of excessive iron loading, and chelation programs with s.c. DFO promote enhanced iron excretion. The goal of management for the patient with thalassemia major is to optimize the balance of these two approaches in order to meet the individual needs of the patient.

Ideally, optimal management can be achieved by the normalization of pre-transfusion hemoglobin levels, the annual monitoring of transfusion requirements, and the assessment of "total" iron balance based on both urinary and stool iron excretion. Our experience has demonstrated that stool iron excretion plays an important role in achieving optimal iron balance and accounts for 60% of the balance at an s.c. DFO dose of 60 mg/kg of body weight, 50% of the balance at a dose of 40 mg/kg, and 30% at 20 mg/kg.

Various factors must be considered when selecting the appropriate dose of s.c. DFO to achieve optimal balance. One must not only assess the dose-response pattern but also consider age, total lifetime iron load, iron saturation, serum ferritin, and organ dysfunction.

The young hypertransfused patient who presents for initial chelation should be maintained at doses to achieve total iron balance but not significant net negative balance. Our experience suggests that a dose of 20–40 mg/kg s.c. DFO administered over an 8-h infusion period for a minimum of five days each week should be initiated at approximately four to five years of age, with a serum ferritin level greater than 1000 ng/ml. Prevention of excessive iron accumulation allows for enhanced organ function, improved growth and development, and prolonged survival.

The older patient with a significant iron burden should be maintained at the higher doses of 40–60 mg/kg s.c. DFO to achieve maximal net negative iron balance. Evidence of advanced hepatic disease and cardiac and endocrine dysfunction warrants consideration of higher doses with intravenous administration.

Our management program has manifested minimal drug toxicity. Daily compliance with DFO treatment regimens remains a significant issue, and techniques to enhance compliance must be developed in order to enhance DFO efficacy.

However, despite variable patient compliance with s.c. DFO and the fact that its efficacious s.c. use began only a little over a decade ago, we are witness to the prolongation of life by this therapy. The future remains even more promising with the ongoing investigations of high-dose intravenous therapy, the search for an oral iron chelator, bone marrow transplantation for some young patients, and the possibility of research efforts toward gene therapy to provide an ultimate cure for all.

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Current Treatment of Cooley's Anemia

Intravenous Chelation Therapy[a]

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Two major themes have run through the discussion of iron chelation therapy at the three previous Cooley's Anemia Symposia. First, with the passage of time, the benefits of regular administration of deferoxamine have become apparent. Recognition of these benefits began with observations on the prevention of further iron accumulation[1] and later included data on the reduction of total body iron stores,[2] depletion of excessive liver iron,[3] prevention of cardiac disease,[4,5] and, in reports published since the last symposium, prolongation of life.[6] The second theme is a steady improvement in the method of administration of deferoxamine. Subcutaneous infusions replaced intramuscular injections,[1,7] enhancing drug efficacy and avoiding the frightening reactions that occurred when deferoxamine was, on rare occasions, accidently injected in high concentration directly into the intravascular space. The addition of intermittent high-dose intravenous infusions of deferoxamine accelerated the rate of iron removal.[2]

These two themes come together in the need to address the problems of an important group of patients who have not received optimal therapy with deferoxamine and who consequently have massive iron stores or iron-induced organ abnormalities such as cardiac failure or arrhythmias. During the past five years, we have studied the use of daily, high-dose, intravenous chelation therapy in these patients. The goals of this form of therapy are to improve compliance, rapidly remove large amounts of iron, and prevent or reverse iron-induced organ dysfunction. In this report, we present the results of this intensive chelation program. We also describe the modifications of the chelation program which are made after iron stores have been reduced to normal levels, and the effect of these modifications on iron balance.

PATIENTS AND METHODS

Patients

Sixteen patients, including six with thalassemia major, nine with sickle cell disease, and one with Diamond-Blackfan anemia, were enrolled in the study. Patients were eligible for the study because of poor compliance with subcutaneous

[a] This work was supported in part by a contract from the Commonwealth of Pennsylvania.

infusions of deferoxamine, very large iron stores, or iron-induced organ dysfunction. One patient, 29 years of age, had not been treated at a thalassemia center previously and was unfamiliar with chelation therapy. Two patients had refused to undertake subcutaneous therapy. The remaining patients had complied poorly with a conventional chelation program of daily (or nightly) subcutaneous infusions.

Methods

In the initial phases of the study, Hickman or Broviac catheters were used for intravenous infusions. Later, subcutaneous ports were placed in newly enrolled patients and in some patients who experienced complications with the externalized catheters.

Deferoxamine was administered at home as a daily 8–12-h infusion at a dose of 15 mg/kg/hr. The total daily dose of 5–12 g was placed in a 20-ml syringe and infused using a battery-powered pump. Patients with subcutaneous ports inserted a new needle into the port every 24–72 h. Ports and externalized catheters were flushed after each infusion with a heparin solution.

Iron stores were monitored by regular measurement of serum ferritin level, serum iron and total iron-binding capacity, and deferoxamine-induced urinary iron excretion. Measurements related to cardiac function included 24-h assessment of cardiac rhythm and examination by echocardiogram, electrocardiogram, and radionuclide scan at rest and with exercise. Routine chemistry studies included assay of serum transaminases, calcium, phosphorus, and glucose. Patients underwent ophthalmologic examination and audiologic testing every six months.

RESULTS

Iron Stores And Organ Function

Initial serum ferritin levels were 1,700–17,409 μg/l. Ferritin levels fell in 14 patients during intravenous deferoxamine therapy, reaching the lowest values thus far after 11–51 months and declining from initial values by 49–99% (FIG. 1). In five patients, ferritin levels fell below 500 μg/l and transferrin saturation, previously 100% in these patients, decreased to 11–77%.

Two patients had clinical cardiac disease prior to beginning daily intravenous deferoxamine. A 30-year-old woman with thalassemia major who had received no prior deferoxamine treatment required digoxin and furosemide for control of heart failure. After 11 months of intravenous chelation therapy, her ferritin level fell from 7,300 to 81 μg/l, and she required no further cardiac medications. Her shortening fraction rose from 23 to 29%. A 20-year-old woman with Diamond-Blackfan anemia had been poorly compliant with subcutaneous deferoxamine therapy and required anti-arrhythmic therapy. Her serum ferritin level fell from 6,068 to 426 μg/l after 26 months of intravenous chelation therapy, and her ventricular arrhythmia resolved.

Three patients, including the two with cardiac disease, had severe hypocalcemia secondary to hypoparathyroidism. The hypocalcemia did not improve despite reduction in body iron stores. None of the sixteen patients developed diabetes or hypothyroidism before or during high-dose chelation therapy.

Catheter And Port Complications

Patients using externalized intravenous catheters experienced local and systemic infections (7 episodes) and catheter obstruction (4 episodes), displacement (3 episodes), and leakage (1 episode). The complication and removal rates were 1 per 1.1 and 1.5 years, respectively. Complications of subcutaneous ports included infection (4), poor access (2), local tenderness (1), and leakage (1). Complication and removal rates were 1 per 3.4 and 5.5 years, respectively.

Modification Of Therapy

The intensity of chelation therapy was reduced in four patients after ferritin levels fell to normal or near-normal levels. In patient with thalassemia major, the

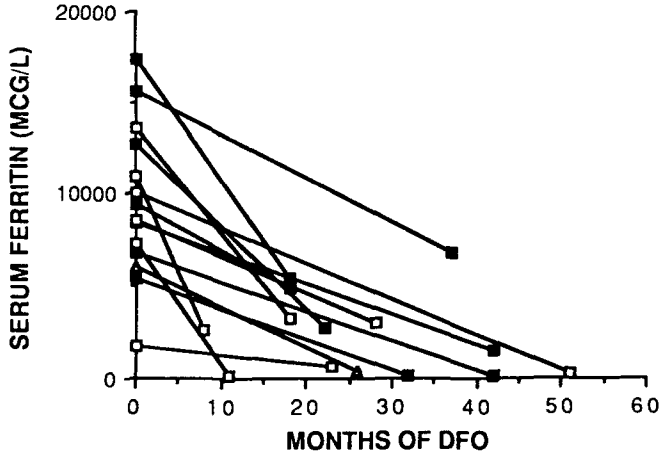

FIGURE 1. Change in ferritin level during intravenous chelation therapy with deferoxamine (DFO). (*Open squares*) Patients with Cooley's anemia, (*closed squares*) patients with sickle cell disease, (*triangles*) a patient with Diamond-Blackfan anemia.

frequency of intravenous infusions of 6 g of deferoxamine was able to be reduced from every day to every other day when her ferritin level fell to 81 μg/l. During the next 14 months, her ferritin level rose to 651 μg/l, and her transferrin saturation increased from 77 to 97%. Her catheter became obstructed, and she began daily subcutaneous infusions of 2 g of deferoxamine (FIG. 2). When her ferritin level rose to 946 μg/l after 14 months using this regimen, her dose was raised to 2.5 and then 3 g. Her serum ferritin level subsequently fell to 497 μg/l after 5 months of subcutaneous therapy at this dose. In another patient with thalassemia major, the frequency of intravenous infusion was reduced from 7 days per week to 3 days per week. Despite the reduction in weekly dose from 42 g to 18 g, the serum ferritin level remained normal (FIG. 3).

Maintenance of low iron stores in two patients with sickle cell disease was assisted by a lower influx of transfusional iron as a result of the substitution of

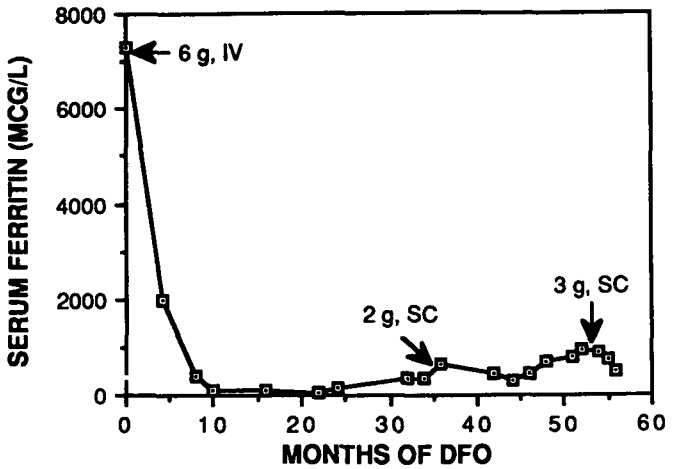

FIGURE 2. Maintenance of low serum ferritin levels in a patient with Cooley's anemia after subcutaneous (SC) infusions with deferoxamine replaced intravenous (IV) chelation therapy.

automated red cell exchange for simple transfusion. In one patient, the ferritin level remained normal after the weekly dose of deferoxamine was reduced from 42 to 18 g. A second patient with sickle cell disease received daily intravenous infusions of 6 g of deferoxamine until his ferritin level fell to 114 μg/l. The dose and frequency of infusion were reduced to 4 g and 3 days per week, respectively. When his ferritin level remained stable after two more months, chelation therapy was stopped. His ferritin level was 37 μg/l 8 months later.

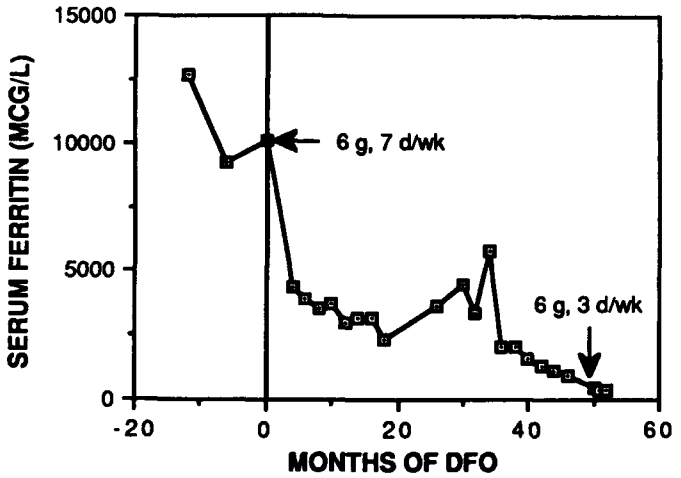

FIGURE 3. Maintenance of normal serum ferritin levels in a patient with Cooley's anemia after reduction in the frequency of intravenous infusions of deferoxamine.

Eye and Ear Abnormalities

One patient had mild macular stippling when first examined 24 months after beginning intravenous chelation therapy at a dose of 6 g or 108 mg/kg/day. Her ferritin level at the time of ophthalmologic examination was 590 μg/l. The eye findings did not change during 42 additional months of intravenous and subcutaneous chelation therapy. A second patient had a 25-dB bilateral, sensorineural hearing loss at 4,000 and 8,000 Hz, which developed during subcutaneous chelation therapy. She subsequently received 21 months of intravenous chelation therapy at doses as high as 121 mg/kg/day without change in her audiogram. The remaining 14 patients have no evidence of drug-induced eye or ear toxicity.

DISCUSSION

The mainstays of therapy for Cooley's anemia remain regular transfusion therapy to maintain a minimum hemoglobin level of at least 9–10 g/dl and iron chelation therapy to prevent the accumulation of excessive iron. Numerous studies suggest that patients treated in this way will have healthier and longer lives.[6,8,9] For young children, early introduction of chelation therapy and good compliance should maintain normal iron stores, preventing the organ damage and early death that were previously inevitable complications of Cooley's anemia. However, older patients with very large iron loads and patients of all ages who fail to comply with conventional chelation therapy need an alternative form of treatment if they are to enjoy similar benefits.

Daily intravenous infusions of high doses of deferoxamine rapidly and safely remove excessive iron. Urinary iron excretion is generally markedly increased in comparison to the levels seen with standard doses of deferoxamine administered by subcutaneous infusions. Ferritin levels return to normal or near-normal levels, accompanied by a decrease in transferrin saturation. Cardiac abnormalities such as congestive heart failure and arrhythmias resolve in some patients as the iron load diminishes. However, other iron-induced organ damage, in particular endocrine dysfunction, may persist despite removal of large amounts of iron.

The major advantages of intravenous chelation therapy are the ability to administer high doses of deferoxamine and the improved compliance that results, in part, from the use of an externalized catheter or a subcutaneous port and the readily apparent effect of therapy on the commonly used measures of iron stores. Earlier studies showed that deferoxamine-induced urinary iron excretion continued to rise as the dose of the chelator was raised from 2 to 16 g.[10] In the current study, iron excretion in response to intravenous deferoxamine was as high as 280 mg/24 h. This large amount of iron excretion causes a rapid fall in the serum ferritin level, giving the patient concrete evidence of the success of therapy and an incentive to continue with daily treatment. Compliance is further enhanced by the avoidance of the local discomfort and swelling associated with subcutaneous infusions. The enhanced iron excretion and improved compliance create a beneficial cycle, leading to the rapid depletion of excessive iron.

Intravenous chelation therapy was unsuccessful in two patients in the present study. One patient, after refusing subcutaneous therapy but agreeing to insertion of a port, complied poorly with treatment; her ferritin level did not change. A second patient used intravenous therapy regularly for several months, then stopped treatment altogether for a similar period; his ferritin levels fluctuated widely in parallel with his use of the chelator. The experience with these patients emphasizes the need

to explore the details of intravenous chelation therapy carefully with patients before inserting a port or catheter.

We are currently investigating several ways of modifying chelation therapy after iron stores return to normal levels. Reduction in the frequency of intravenous infusion gives the patient some relief from the arduous task of daily drug administration and reduces the cost of therapy as the total amount of administered drug decreases. For older patients, a regimen of three infusions of 6 g of deferoxamine each week maintains a normal serum ferritin level. An alternative approach is to use daily subcutaneous infusions to administer the same total dose. This method has the advantage of allowing removal of the port or catheter, but the patient must demonstrate good compliance if the normal iron stores are to be preserved. For patients with sickle cell disease who can be treated with exchange transfusion rather than simple transfusion, the reduction in the rate of transfusional iron loading may make it possible to use small amounts of deferoxamine or stop chelation altogether, watching carefully for evidence of reaccumulation of excessive iron.

The patients treated with high-dose intravenous infusions of deferoxamine in this study did not develop the high incidence of eye and ear abnormalities that has been associated with chelation therapy in other centers.[11-13] This absence of toxicity during intravenous therapy is particularly noteworthy in light of the high doses of drug used and, late in therapy, the low levels of excessive iron observed, two factors that have been identified as contributing to retinal abnormalities and hearing loss. Although drug toxicity was uncommon in this study, the reports of eye and ear complications in other patients provide another reason for reducing the dose of deferoxamine as iron stores return to normal levels.

The rate and type of catheter- and port-related complications with intravenous chelation therapy are similar to those found in other pediatric and adult patients. Infection was the most common problem in the present study. The episodes of local infection or bacteremia were all due to *Staphylococcus* and occurred in 6 of the 16 patients. No infections occurred in patients with Cooley's anemia. As expected, the complication rate with subcutaneous ports was much lower than was the rate with externalized catheters. However, ports must be placed carefully in adolescent girls to avoid problems with access as breast tissue develops.

The best approach to the problem of transfusional iron overload is one of prevention of iron accumulation by early introduction and regular use of deferoxamine. The development of oral chelators may make chelation therapy easier and more readily available for all children with Cooley's anemia. In the meantime, patients who have large iron stores or clinical evidence of tissue damage may still benefit from chelation therapy if iron can be removed quickly and safely. Daily administration of high doses of deferoxamine by intravenous infusion is an effective method for bringing these benefits to patients who will otherwise face early death from iron overload.

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Endocrine Abnormalities in Thalassemia

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Subjects affected by Cooley's anemia who are transfused regularly to a high hemoglobin level have an improved clinical picture and life-span. On the other hand, blood transfusions may transmit viral infections, and they lead to siderosis, which is not always adequately controlled by chelation therapy, one reason being non-compliance with the medical prescriptions. Iron overload, in turn, causes lesions in many organs and parenchymas, and this is the reason why endocrine complications are observed frequently in thalassemics treated by the traditional method of blood transfusions, chelation, and—in some instances—splenectomy.

Several workers[1-5] reported a high incidence of endocrine abnormalities in children, adolescents, and young adults suffering from thalassemia. They demonstrated that these abnormalities were related to iron overload caused by frequent blood transfusions, which mainly affected growth and the pituitary-gonadal axis.

Here we will report data concerning endocrine complications in a series of patients followed in Ferrara. At present, 165 patients with thalassemia major are

regularly followed in our department. In the 1960s, patients were regularly transfused when their hemoglobin levels (Hb) had dropped to 5–6 g/dl. By the end of the early 1980s, this lower limit had gradually been increased to 11.5–12 g/dl,[6] but it has now been decreased to 9.5–10 g/dl, in accordance with the national protocol of treatment. Deferoxamine mesylate (Desferal, Ciba-Geigy) was administered from 1973 to 1979 by intramuscular injection and since 1979 by subcutaneous infusion of 20–50 mg/kg/day nocturnally over 10–12 h as home management, 5–7 days per week. Compliance with chelation treatment was not good in the early 1980s but is now around 90%.

The series of thalassemic subjects followed in Ferrara is peculiar in that the mean age is high. This is due to the combined effect of increased survival and the absence of young patients (FIG. 1). As a consequence of the prevention program, in Ferrara there were only 4 new cases of Cooley's anemia between 1979 and 1983, with none born in the subsequent years; consequently, the majority of the patients are over 16 years of age. Hence, it might be expected that the prevalence of complications due to

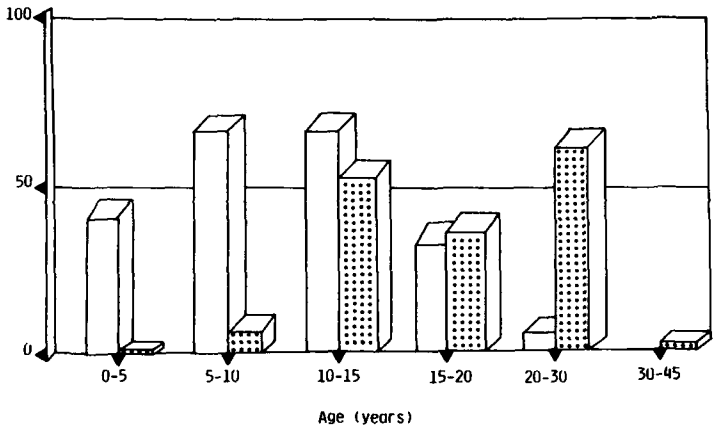

FIGURE 1. Distribution of ages of thalassemic patients followed at the Pediatric Department in Ferrara in 1979 (*white bars*) and 1989 (*dotted bars*).

iron overload such as endocrine abnormalities would be higher here than in other series.

The incidence of endocrine complications in our series is shown in FIGURES 2 and 3. One can see that it varied over the years and appears to correlate with the serum ferritin levels.

INSULIN-DEPENDENT DIABETES MELLITUS

Insulin-dependent diabetes mellitus is frequently a complication of thalassemia major. It severely affects the quality of life of thalassemics, because it requires continuous treatment and adds new fears.

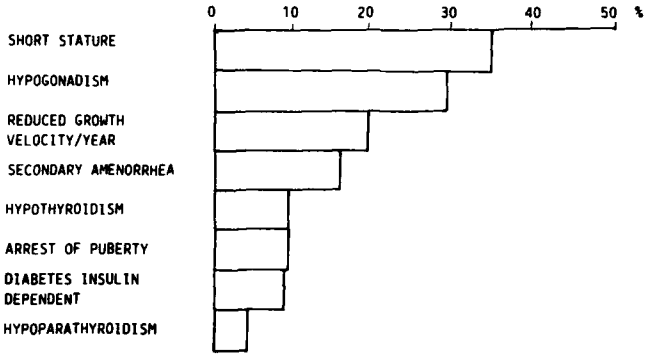

FIGURE 2. Incidence of growth retardation and endocrinopathies in 165 thalassemic patients followed at the Pediatric Department in Ferrara.

An investigation of the prevalence of diabetes mellitus in thalassemics followed in several Italian hospitals was done in 1983.[7] It was found that 29 (13 males and 16 females) of 448 patients (6.4%) were affected by diabetes.

The insulin-dependent diabetes mellitus which complicates thalassemia is thought to be due to iron overload, which damages pancreatic β-cells. However, it is possible that its pathogenesis is more complicated and that other factors may play a part. One such factor seems to be familial in nature. Saudek *et al.*[8] found that 75% of

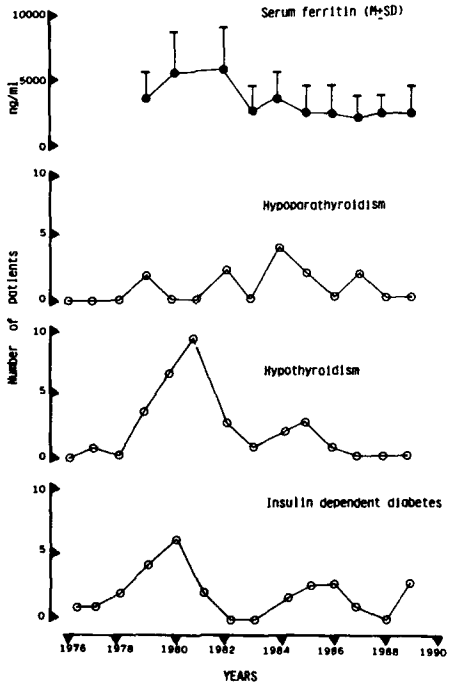

FIGURE 3. Number of endocrine complications in thalassemics in relation to year of onset and to serum ferritin levels (mean ± SD).

thalassemics with a family history of diabetes had impaired glucose tolerance or insulin-dependent diabetes. The importance of constitutional factors was confirmed by us in two series of patients.[7,9] The role of an immunological mechanism seems to be excluded by the absence of anti-islet antibodies.[10]

Another factor which may play a part is chronic hepatitis. Twenty-nine patients (16 males and 13 females) with β-thalassemia major attending the Whittington Hospital (London) and the Divisione Pediatrica in Ferrara were studied in 1986.[9] At the end of the period of observation, the oral glucose tolerance tests had remained normal in 12 patients. Seven of them had normal liver function tests and 5 had chronic active hepatitis documented by liver biopsies. Eleven patients (37.9%) developed impaired oral glucose tolerance. Only 3 of the eleven (27%) had normal liver function tests; 5 had chronic active hepatitis with siderosis grade 3–4, and 3 had cirrhosis with grade 4 siderosis. Six patients (20.6%; 5 males and 1 female) developed overt diabetes requiring insulin treatment. All 6 had liver dysfunction; one had chronic active hepatitis, and 5 had cirrhosis. In addition, patients with the highest serum ferritin levels before subcutaneous chelation therapy most frequently developed abnormal glucose tolerance. This suggests that iron overload is the cause of islet cell damage. However, development of abnormal glucose tolerance or diabetes was also associated with other factors, mainly older age and liver damage.

Hyperinsulinism, the mechanism of which is a matter of controversy, has been found by many other authors and by ourselves in patients with iron overload and/or with cirrhosis.[9,11-14] Merkel *et al.*[15] found hyperinsulinism in pubertal but not in prepubertal thalassemics and suggested that heavy iron overload causes decreased sensitivity to insulin, which is compensated by increased secretion of insulin. Diabetes appears when insulin secretion becomes insufficient to counterbalance insulin insensitivity, because of exhaustion of β-cells, or β-cell damage due to iron deposition, or both these factors. Viral infection may be a third factor involved in the pathogenesis of diabetes mellitus in thalassemics. Among the 29 patients with

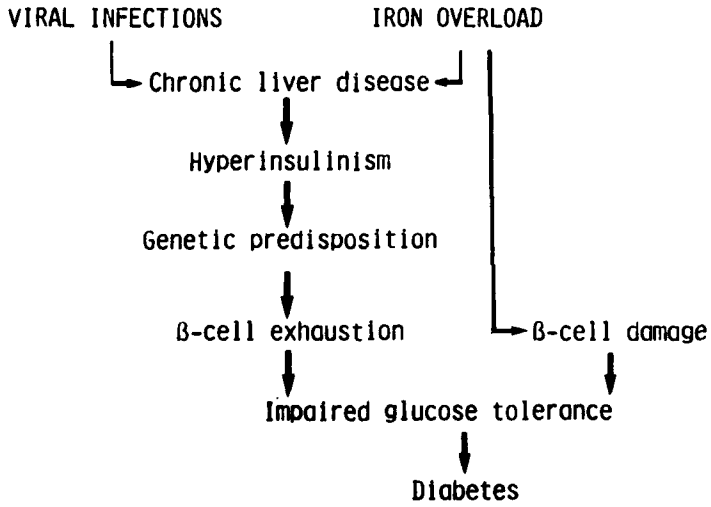

FIGURE 4. Schematic representation of etiopathogenesis of insulin-dependent diabetes mellitus in thalassemia major.

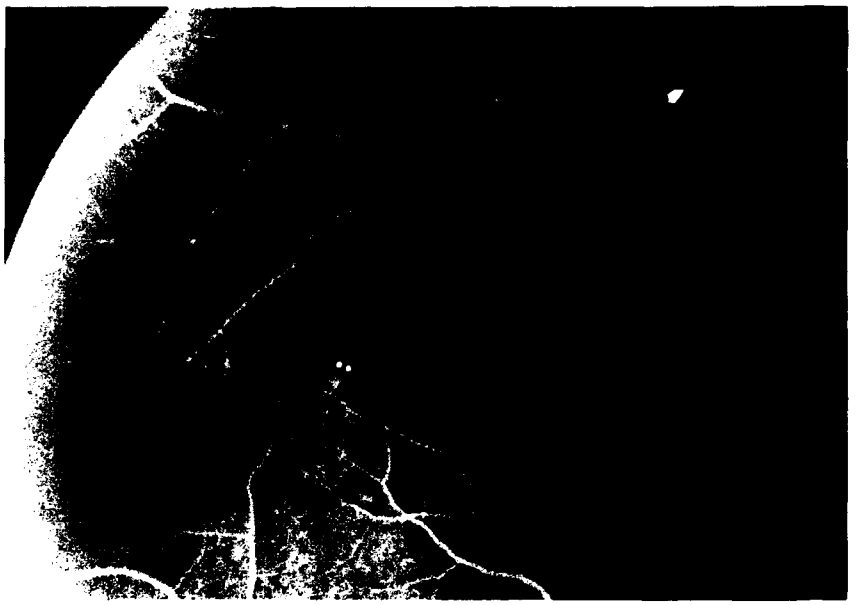

FIGURE 5. Retinofluorangiography showing two microaneurysms in a thalassemic patient with diabetes.

diabetes identified in the Italian investigation mentioned above, the onset of insulin-dependent diabetes mellitus was preceded by acute hepatitis in eight (28%).

In summary, diabetes mellitus which complicates thalassemia major seems to be the consequence of several factors; these are schematically represented in FIGURE 4. Iron overload and viral infections cause chronic liver damage which, in turn, generates alterations in insulin metabolism. At the same time, iron overload causes exhaustion of pancreatic β-cells. The combined effect of these factors together with a constitutional predisposition leads to impaired glucose tolerance and, in time, insulin-dependent diabetes mellitus.

It is known that over a period of years insulin-dependent diabetes mellitus leads to many complications. In the past, the life-span of thalassemics was quite short, and this explains why they did not develop these diabetic complications. As thalassemics receiving good care now survive longer, it might be expected that diabetic complications will appear. Ophthalmoscopic and fluorangiographic examinations were done in a series of 28 thalassemic diabetics (12 males and 16 females) followed in our department whose mean age was 21.1 ± 3.9 years (range, 13.2–28.1 years).[16] The mean duration of the diabetes was 3.6 ± 2.8 years (range, 0–10 years). In stage 1 according to Maione *et al.*,[17] microaneurisms (FIG. 5) were found in three subjects (10.7%). This figure is lower than that found by Burger *et al.*[18] in a large series of non-thalassemic subjects with insulin-dependent diabetes mellitus. More information is needed to confirm that there is a lower incidence of retinal complications in thalassemic diabetics, which could be due to several factors. In thalassemics the duration of diabetes is usually shorter, age at onset is more advanced, pubertal development may be delayed, triglyceride and cholesterol plasma levels are normal or lower than normal,[19] the pathogenic mechanism involved in the development of

diabetes is different, and secretion of contra-insular hormones, for instance of growth hormone (GH), may be impaired due to iron overload (see below, section on growth hormone secretion).

Nephropathy is another frequent complication of diabetes mellitus; this complication accounts for more than 30% of the deaths in type I (insulin-dependent) diabetic patients.[20] Microalbuminuria (assessed by the albumin excretion rate, AER) is considered an indicator of renal damage.[21] We investigated the frequency of elevated AER in 33 thalassemic patients (20 females and 13 males) with insulin-dependent diabetes mellitus. Their ages ranged from 15 to 35 years; their duration of diabetes was 0–10 years. Elevated AER, defined as urinary albumin excretion above 15 μg/min., was found in 17 patients, the majority of whom had poor metabolic control (FIG. 6). Long-term follow-up of these patients may provide information

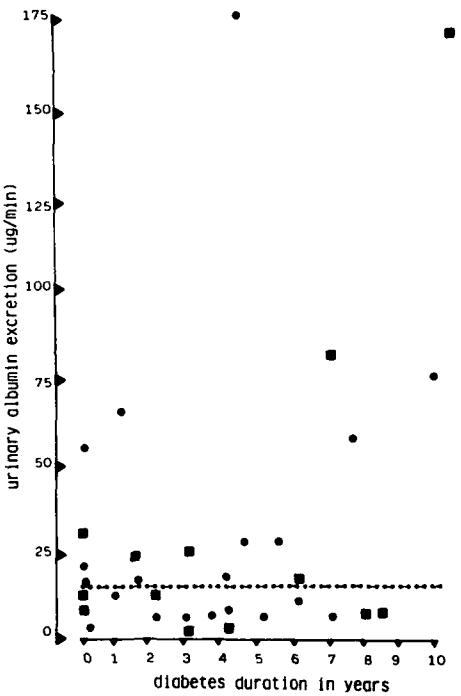

FIGURE 6. Correlation between urinary albumin excretion and duration of insulin-dependent diabetes in thalassemic patients. *Dashed line* indicates upper limit of conventional norm. (■) Males, (●) females.

about their risk of developing nephropathy and the ability of good metabolic control to protect against its progression. As far as we know, this is the first time that microvascular complications due to diabetes have been observed in thalassemics. Our data indicate that we must be prepared to recognize new problems in thalassemics due to their progressively longer survival.

HYPOTHYROIDISM

Data concerning frequency of hypothyroidism in thalassemics are conflicting.[22,23] We first studied the prevalence of hypothyroidism in our series in 1983[23] and found

primary hypothyroidism in 20 out of 114 patients (17.5%) who had been treated by subcutaneous chelation therapy during the previous four years. A high intravenous hemoglobin iron load of at least 0.7 g/kg was found in the majority of hypothyroid patients, emphasizing the etiologic role played by siderosis.

The frequency of hypothyroidism is much lower now than in the past, and no new cases have been observed in our series in the last three years (FIG. 3). In summary, data from the literature and our observations demonstrate that hypothyroidism may complicate the course of thalassemia major and that its frequency may vary from series to series and, within a series, over the course of several years.

HYPOPARATHYROIDISM

Hypoparathyroidism is thought to be the consequence either of reduced parathyroid hormone (PTH) secretion due to damage of parathyroid glands by iron overload or of suppressed PTH secretion induced by bone resorption due to expansion of erythropoietic bone marrow secondary to ineffective erythropoiesis and chronic anemia. Data on the frequency of hypoparathyroidism are conflicting.[24-28] In the last ten years we have observed 24 cases of hypoparathyroidism. In these patients the age at diagnosis ranged from 11 to 24 years (mean 16.5 years), and their serum ferritin levels ranged from 180 to 15,200 ng/ml (mean, 3,772 ng/ml). Hypocalcemia was moderate (serum calcium levels between 6.5 and 8.5 mg/dl) in 21 patients (87%) and severe in three (12%). Signs of hypocalcemia appeared in three patients, two of whom developed tremors and seizures and one of whom developed cardiac failure. At present, 12 patients (7%) regularly followed in our department have hypoparathyroidism. Its onset was preceded or followed in most patients by other endocrinopathies or cardiac complications. This suggests that hypoparathyroidism appears mainly in subjects who have or have had in the past very severe iron overload.

PUBERTAL DEVELOPMENT IN THALASSEMICS

Data concerning pubertal development of thalassemics were collected in northern Italy by Borgna-Pignatti *et al.*[29] in 1983 and 1985. In 1983 these authors found that growth in 62% of the males and 35% of females studied was lower than normal by two standards deviations. Pubertal development was absent in 67% of the males and 38% of the females whose ages were between 12 and 18 years. Growth was slightly improved in 1985. At this time, puberty was absent in 56% of the males (previously 67%) and in 27% of the females (previously 38%). These data show that reduced growth and delayed or absent pubertal development are an important problem in thalassemia, even if the percentage of patients who develop spontaneously is becoming progressively higher.

DELAYED PUBERTY, MENARCHE, AND AMENORRHEA IN THALASSEMIC GIRLS

In females, menarche and menstrual activity are an index of pubertal maturation which is both exact and easy to assess. Data concerning menarche and menstrual activity in thalassemic girls followed in Ferrara are shown in FIGURE 7. It is evident that the percentage of thalassemic girls who reach menarche is progressively

increasing. At the same time, however, there is a parallel increase of the percentage of thalassemic girls who present with secondary amenorrhea. There is an inverse correlation between age at menarche and length of menstrual activity.[30] This is probably to the fact that menarche is more delayed in girls with more severe endocrine impairment.

The endocrine pattern of thalassemics with delayed puberty or primary amenorrhea was studied in our series by De Sanctis *et al.*,[31] who examined 23 females aged from 13 to 29 years. Delayed puberty was diagnosed when there was no breast development by 13 years of age, and amenorrhea when menarche had not appeared by 18 years. Decreased gonadotropin reserves were found in all but one of 23 patients; the gonadal response to human menopausal gonadotropin (hMG) was normal in 14 and reduced in 3 out of 17 subjects with delayed puberty and was more

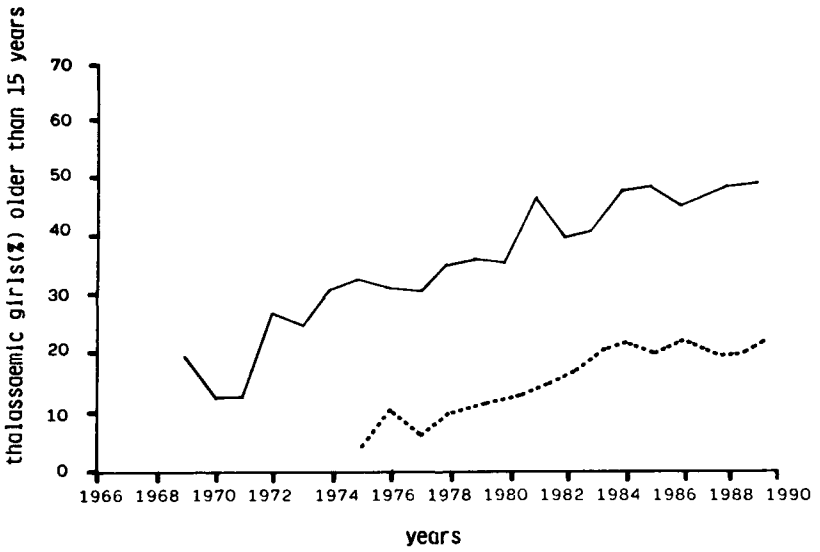

FIGURE 7. Percentage of thalassemic girls with regular menstrual cycles or oligomenorrhea (*solid line*) and with secondary amenorrhea (*dotted line*) from 1969 to 1989.

severely decreased in 4 out of 6 patients with primary amenorrhea. These data suggested that the delayed puberty and primary amenorrhea that complicate thalassemia major can be due to damage of both the pituitary and gonads or of either gland.

The endocrine pattern of patients with secondary amenorrhea was studied in 1988[32] in a series of 8 women whose mean age was 27.3 years and who had developed amenorrhea 2–15 years after their menarche. Basal plasma levels of 17β-estradiol (E_2) were low, with poor or absent response to hMG. Basal serum levels of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) were low with poor response to the gonadotropin-releasing hormone (Gn-RH) test. One patient had an impaired LH and FSH response to the Gn-RH test in the presence of a significant increase of E_2 following hMG stimulation. It is important to note that a

good ovarian response was found in two patients, suggesting the possibility exists of inducing ovulation and pregnancy in these subjects. We had the opportunity of testing this. A 26-year-old woman, who was first transfused at 2 years of age, had menarche at 15 years but at 17 years became amenorrheic. When she got married, the results of the endocrine examinations—Gn-RH test, hMG test, and clomiphene citrate administration—were compatible with a diagnosis of hypothalamic amenorrhea. She wished to have a baby, and ovulation was induced by injection of hMG and human chorionic gonadotropin (hCG).[33] A single pregnancy was started, which was uneventful until 35 weeks of gestation, when a cesarean section was performed. This baby is normal and well. Two women with secondary amenorrhea who did not respond to the short hMG test had significant elevations of serum E_2 and the development of a dominant follicle after prolonged hMG administration. However, whether pregnancy can be induced under these circumstances remains to be determined.[34]

In summary, our findings indicate that both pituitary and gonadal damage may be responsible for the secondary amenorrhea observed in thalassemic females. The iron overload, present in all patients, may play a part in endocrine dysfunction. The high prevalence of ovarian failure in our patients may be an age-dependent phenomenon. Associated factors, such as chronic liver disease and diabetes mellitus, which were present in some of our patients, may contribute to secondary amenorrhea. Endocrine evaluation of thalassemics with delayed puberty or primary amenorrhea is important, because the appropriate treatment can then be selected.

SPERMATOGENESIS IN THALASSEMIC MALES

Virtually nothing was known about spermatogenesis in thalassemic males who had reached full pubertal development. We did sperm analysis and hormone assays in a group of 26 fully mature thalassemic patients, whose ages ranged from 15 to 25 years.[35] Normal sperm count and motility were found in only 12 patients (46%), while 14 (53%) had low total sperm count and/or motility. There was no correlation between either total sperm count or sperm motility and serum ferritin levels. This suggests that iron overload which is not severe enough to prevent sexual development does not impair spermatogenesis. On the other hand, it was found that three out of four patients with serum ferritin levels lower than 500 ng/ml had poor sperm motility. These findings have been confirmed in a larger group of patients (FIG. 8). This suggested to us that reduced sperm motility may be the result of deferoxamine toxicity, which appears only when iron stores are depleted.

HYPOGONADISM IN THE THALASSEMIC MALE

In the thalassemic male of pubertal age, hypogonadism manifests itself with a clinical picture of delayed or arrested puberty, usually due, in the majority of patients, to gonadotropin deficiency or, less frequently, to gonadal endocrine failure.[36] If untreated, these patients cannot have a normal sexual life or reproduce.

Gonadotropin treatment can induce pubertal development and spermatogenesis. We treated 10 gonadotropin-deficient thalassemics, 15–23 years old, with exogenous gonadotropins for 1–4 years.[37] After hCG administration, increased testicular volume

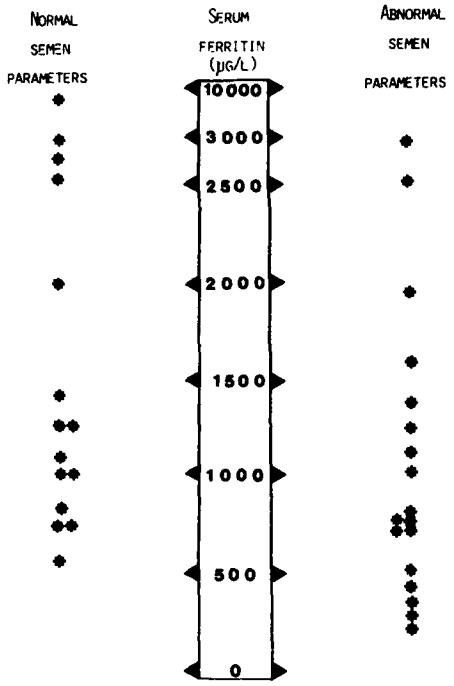

FIGURE 8. Relation between serum ferritin levels and seminal parameters in 33 thalassemic males with full pubertal development.

was observed in all patients. Seven patients produced sperm during hCG treatment given for 6–14 months. hMG was added to the hCG regime in 8 patients. Testicular volume increased further in five of the eight patients. In five patients, total sperm count and motility increased further. One patient was married, and after 3 months of therapy his wife became pregnant. His ferritin levels had been reduced by chelation from 10,750 to 105 ng/ml. Treatment was stopped; but he maintained normal seminal parameters, and his wife became pregnant again two years later (FIG. 9). Secondary hypogonadism developed after 2 years, when he was 29 years old and his serum ferritin level was 1,430 ng/ml. On the other hand, three patients remained azoospermic despite continuing hCG and hMG therapy for 6–18 months. A positive effect of this treatment on growth was observed in all but one patient. This is in agreement with the observations of Bozzola *et al.*[38]

GROWTH HORMONE SECRETION

Growth hormone (GH) secretion in thalassemics has been found by several authors[2,3,39,40] to be normal or slightly decreased. On the other hand, Pintor *et al.*[41] demonstrated decreased GH response to insulin and clonidine. In most of their patients, there was also an impaired GH response to growth hormone–releasing hormone (GH-RH). According to Leger *et al.*,[40] this apparent discrepancy may be

due to the different ages of the patients. This indicates that pituitary and/or hypothalamic dysfunction may be an age-related phenomenon in thalassemic patients.

We investigated the 24-h profile of serum GH secretion in six patients with thalassemia major and one with thalassemia intermedia. In these patients we also assessed the response to GH-RH (1 μg/kg body weight, intravenously administered). Analysis of mean 24-h levels, peak amplitudes, and peak frequencies demonstrated that GH secretion was significantly decreased in two patients, 12.1 and 16 years old. In the second patient the GH response to GH-RH was impaired, suggesting either a pituitary lesion or a lack of pituitary stimulation due to hypothalamic dysfunction (FIGS. 10 and 11).

Sukegawa *et al.*[42] have reported that nocturnal urinary GH excretion significantly reflects the nocturnal endogenous GH secretion rate. Furthermore, the nocturnal urinary GH values correlate with the 24-h mean plasma concentration and 24-h urinary GH values. We determined urinary GH in 38 thalassemics, aged 7–24 years, with short stature or reduced growth velocity. GH was determined by sensitive

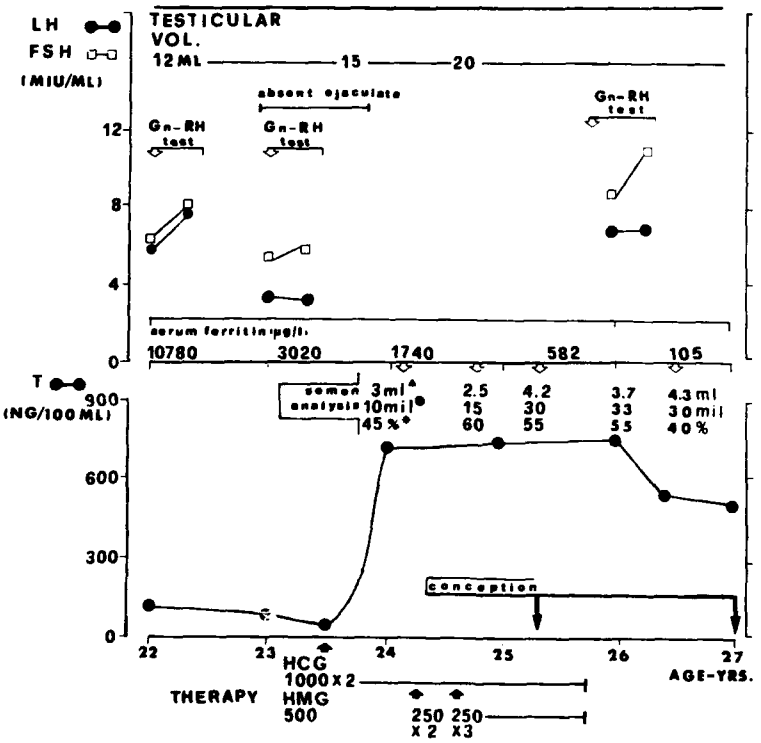

FIGURE 9. Clinical and laboratory findings and treatment in a thalassemic male with secondary hypogonadism. Serum analysis: LH (●: **upper panel**), luteinizing hormone; FSH (□), follicle-stimulating hormone; T (●: **lower panel**), testosterone. Testicular volume, total sperm count, and sperm motility are indicated.

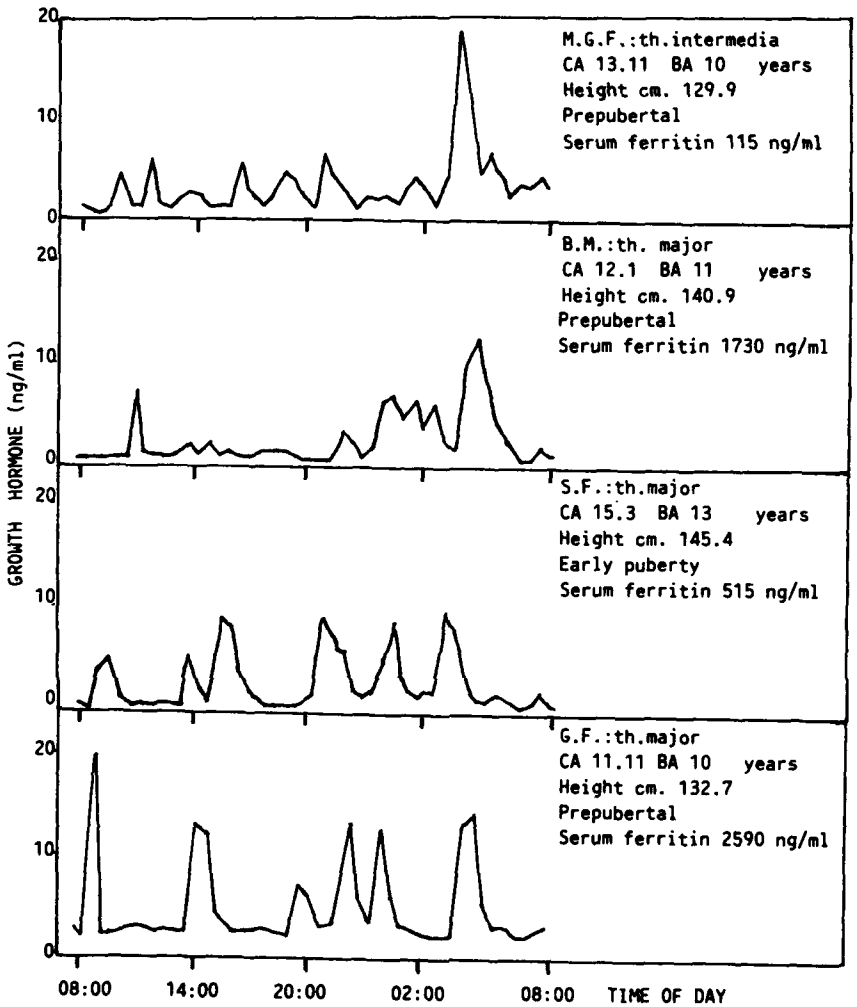

FIGURE 10. Representative growth hormone secretory dynamics in male patients with thalassemia (th.) major or intermedia. CA, chronological age; BA, bone age.

enzyme immunometric assay (EIA) on the early morning urine from three consecutive days. GH amounts comparable to those found in deficient children were found in 12 patients, that is, in two of 10 subjects 7–12 years old (20%), in seven of 15 subjects 12–15 years old (40%), and in 3 (25%) of 13 subjects older than 15 (FIG. 12). The high incidence of reduced GH excretion in the peripubertal and pubertal group may be due either to GH deficiency or to delayed puberty. If further investigations confirm our data, GH urinary measurements could prove to be a simple screening method for subjects with suspected GH deficiency.

ADRENAL FUNCTION

Adrenal insufficiency was observed in thalassemics[39,43] in the past. Recently, Sklar *et al.*[44] found normal cortisol and reduced adrenal androgen secretion. We found normal plasma cortisol, plasma ACTH and urinary free cortisol levels in 22 subjects, aged from 11 to 24 years (mean age 13.7 ± 3) (FIG. 13).

MULTIPLE ENDOCRINE ABNORMALITIES

In our series, 6 out 165 (3.6%) thalassemics developed multiple endocrine abnormalities. In other diseases with multiple endocrine abnormalities, the etiology was thought to be autoimmune. Therefore, Di Palma, Volta, Molinaro, De Sanctis, Bianchi, and Vullo assayed for the presence of organ-specific and non-specific antibodies for 23 thalassemics with polyendocrine failure, including diabetes, primary hypothyroidism, hypoparathyroidism, and hypogonadism. No evidence of

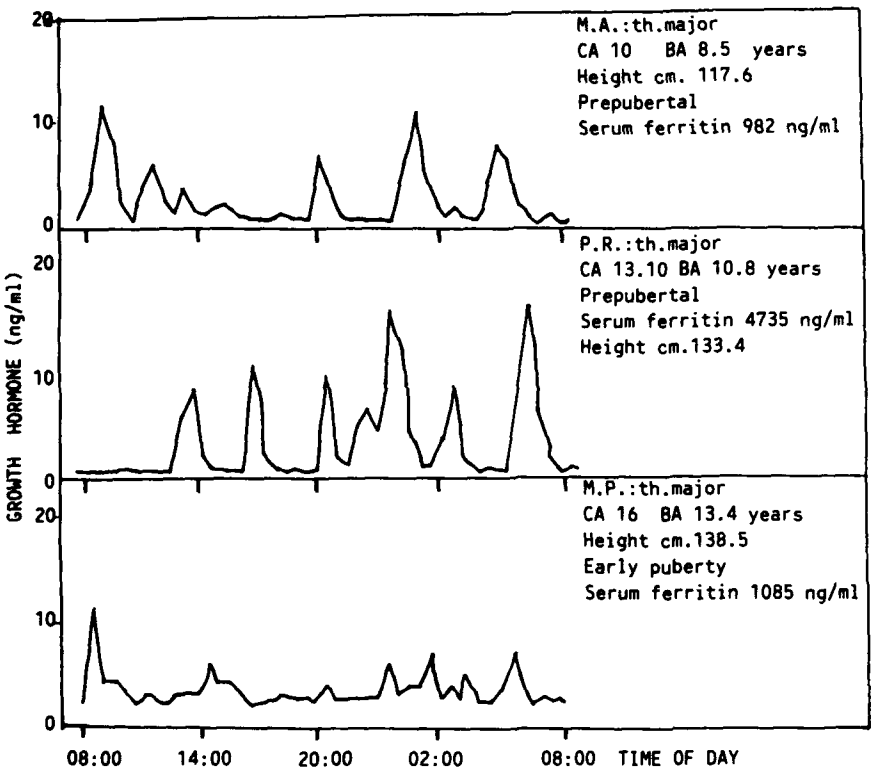

FIGURE 11. Representative growth hormone secretory dynamics in female patients with thalassemia (th.) major. CA, chronological age; BA, bone age.

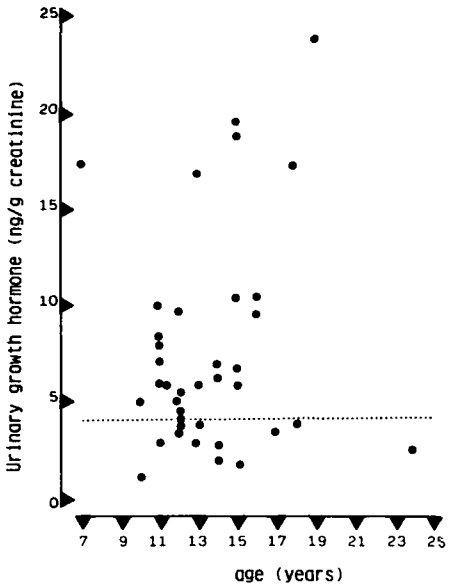

FIGURE 12. Mean of growth hormone values in the early morning urine taken on three consecutive days in 38 thalassemic patients. (*Dotted line*) Lower limit of normal below which a diagnosis of GH deficiency should be considered.

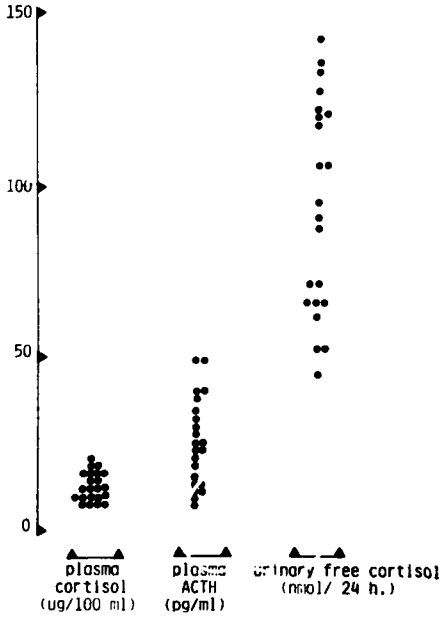

FIGURE 13. Plasma cortisol, plasma ACTH, and urinary free cortisol in 22 patients with thalassemia major.

immunological processes leading to polyglandular involvement was found (data not shown).

PROBLEMS RELATED TO TREATMENT OF ENDOCRINE COMPLICATIONS IN THALASSEMICS

Treatment of endocrine complications in thalassemics presents peculiar difficulties for several reasons. First of all, it makes the therapeutic regime heavier. This is a cause of further discomfort to the patients and may encourage medication non-compliance, since compliance is inversely related to the complexity of the therapeutic regime and to the number of prescriptions.[45] Secondly, the hormones which it is necessary to administer to compensate endocrine deficiencies may adversely affect other complications present in the patient. This is the case, for instance, with sex hormones given to thalassemics with liver lesions or diabetes mellitus. Thirdly, the commencement of substitutive hormone administration at a young age may enhance the adverse side effects of this type of therapy. This is the case, for instance, with estrogen and progestin administration to thalassemic females with primary or secondary amenorrhea.[46] In summary, the evaluation of the pros and the cons of hormone administration, which may be difficult in non-thalassemic individuals, may be even more difficult in thalassemics. This adds new arguments in favor of the necessity of preventing endocrine complications before they develop.

CONCLUSIONS

Endocrine dysfunctions frequently complicate the course of β-thalassemia major. This has been confirmed in our series of patients, in which we have observed many endocrine dysfunctions due to iron overload and possibly associated with other factors, such as chronic liver disease, viral infections, and constitutional predisposition. The incidence of the various endocrinopathies may be higher in our series than in others for different reasons, one of them being the high mean age of the patients we studied. We observed also that the incidence of endocrine complications may vary over a period of years and that the same clinical picture may be the consequence of damage to different endocrine glands. In thalassemics the simultaneous involvement of more than one endocrine gland in the complications is very common, which is different from the finding in non-thalassemics. This, added to the simultaneous involvement of liver and other organs, makes the treatment of endocrine complications in thalassemics more difficult than usual. Endocrine complications may be the cause of immense embarrassment to patients and of great concern to their patients; and it may be a cause of premature death. As a consequence, endocrine functions must be checked periodically in thalassemics to detect endocrine disturbances as early as possible so that they can be treated properly. Patients with endocrine complications must be followed meticulously, because of the possibility of the development of new complications, such as microvascular diabetic disease, secondary to endocrine abnormalities.

The frequency of some endocrine complications is diminishing in our series. Hence, it is possible to be optimistic for the future and to hope that improved treatment will prevent parenchymal damage and the consequent endocrine abnormalities.

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Oral Iron Chelators for the Clinical Management of Iron Overload

Current Hopes and Problems[a]

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In the 1960s, at the same time that hypertransfusion was introduced for the management of Cooley's anemia,[1] the concept of iron chelation therapy was also inaugurated with deferoxamine.[2] As a chronic anemia was transformed into a disease of iron overload, some relief was predicted by the newly acquired possibility of enhancing iron excretion. A quarter of a century later the survival of patients with Cooley's anemia has extended well into the third decade, for those who have been able to comply with a rigorous transfusion program and an assiduous chelation therapy.[3] Compliance, however, has not been easy: these therapies are complex and expensive and thus not available to many patients throughout the world. Even where available, however, the chelation arm of the therapy is cumbersome and difficult to tolerate, particularly for the teenagers who share the zest for life appropriate for their age. Similar problems are encountered also by other patients with transfusion-induced hemosiderosis, such as, for instance, patients with sickle cell syndrome who have suffered a stroke.

At the symposium on which this volume is based we have heard again that Cooley's anemia can be cured by replacing the bone marrow with that of an unaffected sibling.[4] Thus, there is light at the end of the tunnel: Cooley's anemia is curable. Unfortunately this curative approach is available only for those patients with a compatibly matched donor, and it is best tolerated by those who are youngest and in the best overall clinical condition. For the vast majority of the patients, therefore, a cure of the disease must await the realization of "gene therapy." While at this meeting incredible advances have been reported,[5] the application to humans remains a promise for the future. Yet, although this goal appears nearer every day, from the patients' perspective, progress appears tantalizingly slow.

It has become quite clear that chelation therapy is life-saving, and it is necessary even for the success of bone marrow replacement.[6] In 1990, the patient with Cooley's anemia, while anxiously awaiting a hopeful future, has to contend with the difficulties, both economic and pragmatic, of subcutaneous deferoxamine treatment: not all can afford them. When "genetic engineering" becomes available, it would be tragic if the clinical status of the patients were so deteriorated that they could not avail themselves of such a cure. It thus remains critical today to keep the patient in the best shape. An oral iron chelator appears needed now more than ever, since Cooley's anemia is curable. The cure is imminent, but only for those survivors who are well transfused and chelated.

The need and the hopes for an oral chelator have been with us for a long time.

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Even before the demonstration by Propper *et al.*[7] that deferoxamine by prolonged infusion resulted in increased iron excretion and may lead to a negative iron balance, the urgency of developing an oral chelator was perceived by the scientific and political communities. In the United States, as soon as the National Cooley's Anemia Control Act was signed into law in 1972, the Inter-Agency Coordinating Committee on Cooley's Anemia identified the development of efficient oral chelators as the first priority.

In September 1975, the First Symposium on the Development of Oral Chelators for Clinical Use was held at the National Institutes of Health (NIH).[8] In August 1980, the second such symposium was held in San Francisco.[9] The proceedings of these meetings show a road littered with hopes, false starts, and illusions. But the scientific community did not give up; by the end of the 1980s several promising compounds appeared at the horizon. At a small group meeting held in February 1989 at the NIH under joint sponsorship with the Cooley's Anemia Foundation the "state of the art" of oral iron chelation was reviewed.[6] It appeared clear that we now have at least four promising families of compounds: pyridoxal isonicotinoyl hydrazone (PIH) and its derivatives, the hydroxypyrid-ones, desferrithiocin, and hydroxybenzyl-ethylendiamine-diacetic acid (HBED) and its dimethylester.

Does this mean that the quest for the oral iron chelator is finally ended? Can the patients get ready to throw away pump-syringe, needles, and related paraphernalia? The answers to these questions are mixed. There are two major requirements for an oral chelator: efficacy and safety. None of the present candidate drugs has, as yet, passed all the tests. It must be realized that what we ask of an oral chelator is formidable, since the successful drug must be administered to the patients for a lifetime without toxicity; and, on the other hand, deferoxamine, the drug to be replaced, has an extraordinary record of safety and efficacy.

TABLE 1. U.S.A. Food and Drug Administration General Guidelines for Animal Toxicity Studies (Synopsis)

Duration of Human Administration	Phase	Subacute or Chronic Toxicity Tests
Up to 2 weeks	I, II, III	2 Species; 2 weeks
Up to 3 months	I, II	2 Species; 4 weeks
	III	2 Species; 3 months
6 months to unlimited time	I, II	2 Species; 3 months
	III	2 Species; 3 months or longer

The development of a new drug is covered in the United States by certain rules put forward by the Food and Drug Administration (FDA) to protect the patients. Preliminary to human use, certain toxicity studies in animals should be completed (TABLE 1); these requirements are reasonable and not really excessive. Similar requirements exist in other Western countries (for instance, in the United Kingdom, from the Department of Health and Social Security—DHSS). Of the four main drugs, two have completed these requirements, PIH and HBED. For the two others, hydroxypyrid-ones and desferrithiocin, only partial data from animal studies are today available. Without such studies, human clinical trials cannot be started in the United States (and probably should not be started in other countries).

Of the two drugs for which toxicity studies are completed, PIH has undergone preliminary clinical trials without evidence of toxicity, but also without a great record

of efficacy. For HBED, while the animal toxicity studies have been completed, this compound has not yet been submitted for FDA approval of human trials, in part because its main developer, Dr. R. W. Grady, expects one of its dimethyl-ester derivatives which promises greater efficacy and little toxicity, to become available soon.

Desferrithiocin is still undergoing animal toxicity studies. There is concern that its safety, acceptable when iron overload is markedly present, may be unacceptable when iron overload is decreased.

The hydroxypyrid-one group of drugs appears, as of today, the most promising. One of these compounds, the 1,2-dimethyl-3-hydroxypyrid-one (also called L1) has undergone preliminary human trials.[10] Unfortunately, these trials have not been preceded by animal toxicity studies accepted by either the United States FDA or the British DHSS. The clinical studies in humans have been conducted under the approval of a local hospital review board on the assumption that patients non-compliant with deferoxamine, and thus with extremely high iron load, are at risk of life and therefore may be subjected to a treatment that has been only suboptimally evaluated. Many investigators, at least in the United States, would disagree with such views; they would recommend that those patients be treated instead with intensive intravenous deferoxamine, as used successfully by several groups in this country[6] and reported at this conference as well (see paper by COHEN ET AL.).

In any event, the preliminary clinical trials in humans suggest that L1 is effective. Many investigators, however, remain perplexed by the observation that, at least in the trial in London, an alleged very large iron excretion has not resulted in a decrease in serum ferritin, even after months of treatment. On the other hand, in a controlled short-term Canadian trial, excretion of iron was observed; but it was not much different from that induced by deferoxamine.[6]

The major controversy on the widespread use of L1 has been, however, generated by concern about its potential toxicity. Some animal studies have suggested the presence of bone marrow toxicity,[11] but other studies have denied it.[12] At least in one patient, severe neutropenia was clearly related to drug intake.[13] This developed in a patient with Blackfan-Diamond anemia, a condition not usually associated with neutropenic crisis. Additional animal studies have suggested other possible toxic effects, including adrenal enlargement. Toxicity for the related compound CP94 (2-ethyl-hydroxypyrid-one) has also been observed in animals.

It is difficult to assess the relevance of these animal toxicities, often observed at very high doses, to human use of these drugs. While it is unrealistic to expect any drug to exhibit no animal toxicity, regardless of dose, the observed effects should guide the human trials to the appropriate safeguards, if these are to be successful. The promises of the various hydroxypyrid-ones are too important to be discarded. On the other hand, careless trials may result in unpredictable problems that may be difficult to interpret, and there is the risk that a potentially useful drug may be prematurely abandoned. It is clear that a more rigorous approach is necessary. But, while a more cautious attitude appears desirable, the recent editorial in the *Lancet* that urged a total halt to any additional trial of L1 in humans appears to be just as hasty as the premature clinical trials it condemned.[14]

In an attempt to bring a rigorous scientific approach to this field, away from local controversies, and at the urging of the Cooley's Anemia Foundation, the Fe-chelator testing program of the NIH National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) is completing animal toxicity studies of L1. Additional toxicity studies are also being performed on L1 by Dr. R. W. Grady and on C94 by Dr. G. M. Brittenham. The Fe-chelator testing program of the NIH NIDDK is also testing desferrithiocin. Animal toxicity studies have been completed for HBED and PIH. As a result of these concerted efforts, by the time this volume is published, several compounds should be ready, with FDA approval, for preliminary human short-term

trials. From the results of these, the best drugs will be chosen for chronic animal toxicity testing, to be ultimately followed by detailed cooperative multi-institution long-term human trials. The commitment of the two NIH Institutes (the NIDDK for the animal studies and the Heart, Lung and Blood Institute [HLBI] for the clinical trials) testifies to the importance and urgency of the assessment of these potential drugs. It is hoped that with this more scientific approach an effective oral chelator may emerge that could be given to the patients in a controlled fashion, without untoward risks.

In the meantime, attention should be focused on the recent studies of high-dose deferoxamine[6] and its ability not only to prevent, but also to reverse, the cardiotoxicity of iron.[15] At this point, the patients should again be asked to have patience and wait until the present confusion is clarified and a winner emerges among the various potentially contending drugs.[16]

But the future appears bright: the present symposium is quite different from all the previous ones, as the goal of an effective and safe oral chelator appears finally within reach.

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Pyridoxal Isonicotinoyl Hydrazone

Effective Iron Chelation after Oral Administration[a]

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INTRODUCTION

Both the quality and the length of life of patients with Cooley's anemia could be substantially improved by the development of a safe, inexpensive chelating agent that effectively promotes the excretion of iron after oral administration. Without therapy, potentially lethal amounts of iron accumulate in patients with refractory anemia who require regular blood transfusions, absorb excessive amounts of dietary iron, or both. Because the body lacks an effective means to eliminate excess iron, the iron contained in transfused red cells or derived from dietary absorption is progressively deposited in the liver, heart, pancreas, and other organs. Cirrhosis, heart disease, diabetes, and other disorders develop; death is usually the result of cardiac failure. New means of treating and preventing these complications of iron overload may soon become available with the continued development of several orally active iron-chelating agents that are now in or near clinical trial.[1-6] The results of preclinical and clinical studies with one of these agents, pyridoxal isonicotinoyl hydrazone (PIH), will be reviewed here. Both the distinctive pharmacokinetic profile of PIH and the extensive experience in the use of its constituents, isoniazid (INH) and pyridoxal (vitamin B_6), in the chronic therapy of tuberculosis suggest that this chelator may be a safe and non-toxic agent that is particularly well suited for prolonged use in the treatment of iron overload in Cooley's anemia.

PYRIDOXAL ISONICOTINOYL HYDRAZONE: PRECLINICAL STUDIES

Discovery That PIH Is an Iron Chelator

In the period from 1954 to 1959, isonicotinoyl hydrazide (isoniazid, INH) and both pyridoxal[7] and pyridoxal-5-phosphate[8] were used to prepare pyridoxal isonicotinoyl hydrazone (PIH). PIH was found to exhibit coenzymatic activity toward some pyridoxal-5-phosphate–dependent enzymes,[9,10] but this phenomenon was eventually found to be simply the result of a slow dissociation of the hydrazone, leading to the regeneration of free pyridoxal-5-phosphate.[11] The iron-chelating property of PIH was not recognized until Ponka and co-workers[12] serendipitously observed that the addition of pyridoxal-5-phosphate or pyridoxal to ^{59}Fe-loaded reticulocytes incu-

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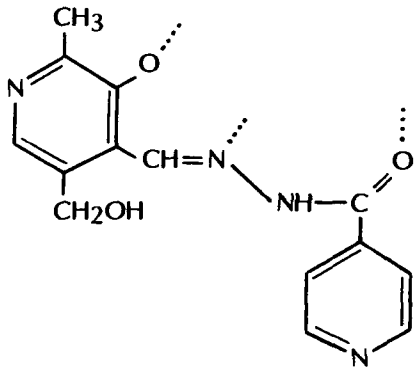

FIGURE 1. Schematic representation of the structure of pyridoxal isonicotinoyl hydrazone (PIH) as a tridentate ligand as proposed by Huang and Ponka,[22] with potential coordination positions for iron chelation shown by *dotted lines*.

bated with INH to block ^{59}Fe utilization for heme synthesis caused the release of a considerable amount of radioiron from the cells. Investigation of this phenomenon demonstrated that the mobilization was the result of the formation of PIH, which in turn chelated the iron in solution.

Synthesis, Structure, and Properties of PIH

PIH may be readily synthesized by the Schiff-base condensation of INH with the 4-aldehyde group of pyridoxal or pyridoxal-5-phosphate[12,13] (but it cannot be synthesized from INH and pyridoxine, which lacks the aldehyde group). The PIH is obtained as an *E-Z* isomeric mixture. The photosensitive isomer (*Z*, colorless to bright yellow) converts in the solid state into the *E* isomer on exposure to daylight at room temperature.[14] PIH prepared in this fashion is a brilliant orange-red powder with a slightly bitter taste. In aqueous solution PIH is bright yellow; the absorption spectrum shows peaks at 342 and 400 nm. The free-base form of PIH has the formula $C_{14}H_{14}N_4O_3$ and a predicted molecular weight of 286 that has been confirmed by mass spectrometry.[14] PIH has a melting point of 255°C. Maximum solubility at neutral pH is 6 mmol/l; at a pH of 7.0, solubility varies between 6 mmol/l at 0°C and 6.8 mmol/l at 37°C.[14,15]

Efficacy of PIH As an Iron Chelator In Vitro

Investigation of iron-binding by PIH *in vitro* showed that the drug optimally formed a 2:1 molar chelate with Fe^{3+}; the molecular weight of the Fe-PIH_2 complex is 628.[12] For comparison, desferrioxamine (molecular weight, 657) complexes with iron in a 1:1 molar ratio to form ferrioxamine, with a molecular weight of 713. Thus, 1.0 g of PIH and 1.15 g of desferrioxamine bind an equivalent amount of iron, about 0.1 g. Experiments with a number of compounds related to PIH suggested that the phenolic group, the double-bonded nitrogen, and the oxygen atom of the carbonyl group participate in metal binding.[15,16] PIH thus appeared to function as a tridentate ligand. The structure of PIH[12] and the model of the Fe-PIH_2 complex[16] proposed by Ponka and colleagues are shown in FIGURES 1 and 2, respectively. Spectrophotometric studies of mixtures of PIH and iron salts to determine the stability constant, *K*, of the Fe-PIH_2 complex indicated that log $K = 8.7$, with a maximum value obtained at

pH 5.5 in methanolic solutions.[14] For comparison, transferrin has a log K of 24.0[17] and desferrioxamine, of 30.6.[18] The Fe-PIH_2 complex breaks down as the pH of the solution reaches a value of about 2. On the alkaline side, the complex is much more stable and can be detected at pH values as high as 12.[14] The binding of other metal ions by PIH has also been investigated. Incubation of 1 mmol/l solutions of PIH with an equimolar concentration of metal salts for 24 h with monitoring of the spectra[15] showed that complexes were formed with Cu^{2+}, Zn^{2+}, Co^{2+}, and Pb^{2+} but not with Mg^{2+}, Ca^{2+}, Mn^{2+}, or Cd^{2+}. PIH readily enters and chelates iron from cells in culture systems with Chang cells, fibroblasts, and hepatocytes.[19,20] Furthermore, the Fe-PIH_2 iron chelate can donate iron to cells in culture.[21]

A series of studies by Ponka and co-workers using rabbit reticulocytes labeled with ^{59}Fe have provided insight into the mechanism of iron chelation by PIH at the cellular level.[16,22] In these experiments, reticulocytes isolated from rabbits treated with phenylhydrazine are incubated with ^{59}Fe-labeled rabbit plasma and INH to block the utilization of intracellular non-heme iron for heme synthesis. The labeled reticulocytes are then incubated with chelators, and the extent and site of iron mobilization are determined. Desferrioxamine does not seem to enter reticulocytes and has little activity in this preparation. Ponka and co-workers first discovered the iron-chelating property of PIH in this system.[12] The entry of PIH into the reticulocyte did not seem to depend upon a specific transport mechanism; instead, PIH appeared to simply diffuse across the erythroid membranes in a rapid, energy-independent

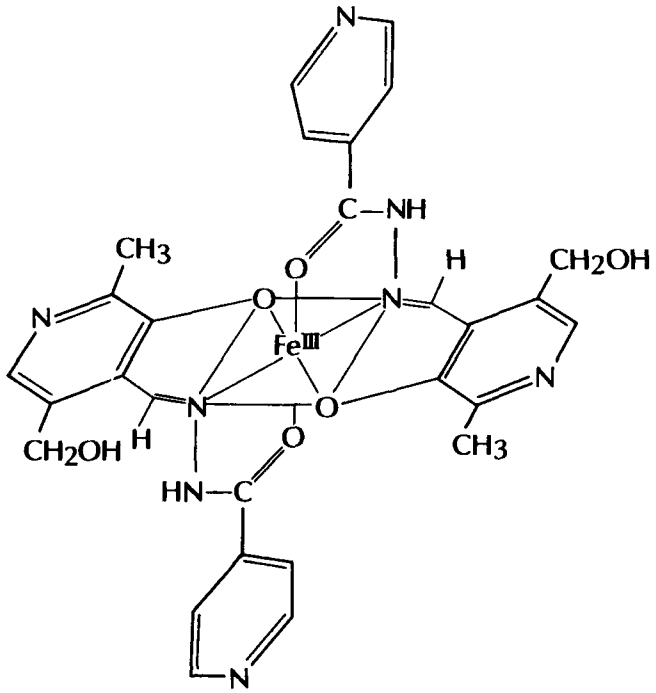

FIGURE 2. Schematic representation of the structure of the pyridoxal isonicotinoyl hydrazone–iron complex (Fe-PIH_2) proposed by Ponka and colleagues.[16] Other possible mechanisms of chelation have been suggested by Johnson and colleagues.[29]

manner. In a mitochondrial preparation, iron derived from a source in the ferrous state was most readily bound by PIH. The exit of the Fe-PIH_2 complex from the cell required energy and could be inhibited by disruption of the microtubular lattice with vinca alkaloids. Either energy inhibition or microtubular disruption resulted in the accumulation of labeled iron in a non-transferrin, non-heme, non-ferritin ethanol fraction of the reticulocyte, presumably as Fe-PIH_2. Thus, these results provided evidence that the mechanism of PIH action in the reticulocyte system involves at least three stages: (1) passive diffusion of PIH into cells, (2) chelation of iron derived mainly from a ferrous iron source within mitochondria, and (3) active extrusion of the Fe-PIH_2 complex via an energy-dependent process requiring an intact microtubular system. These studies indicated that PIH functions as an effective chelator in the reticulocyte system not only because of its ability to complex with iron, but also because of its ability to rapidly pass through the membrane barriers of the cell by passive diffusion and, after the chelate is formed intracellularly, to harness an energy-dependent cellular process to eject the Fe-PIH_2 from the cell along the microtubular system. Further studies of the effect of PIH and a variety of analogues on iron release from macrophages, reticulocytes, and hepatocytes have recently been published.[23,24]

Efficacy of PIH As an Iron Chelator In Vivo

Ponka and co-workers were the first to examine the activity of PIH *in vivo*.[16,25] Initially, normal (not iron overloaded) rats, injected 2–3 days previously with ^{59}Fe-transferrin to label the iron contained within hepatic parenchymal cells, were given intraperitoneal injections of saline or desferrioxamine after bile duct cannulation. In a similar experiment, rats were given heat-damaged ^{59}Fe-loaded erythrocytes, to label reticuloendothelial iron stores, before intraperitoneal injection with saline or PIH. The results of these experiments suggested that PIH takes up iron from hepatocytes and enters bile as an Fe-PIH_2 complex. After intraperitoneal injection, the effect of PIH begins promptly. As early as 10–15 minutes after administration, Fe-PIH_2 is detectable in the bile; and the peak of excretion is seen within 1–5 h. The time-course of excretion after an injection of desferrioxamine is similar, but the amount of iron excreted is somewhat less than that found after a similar dose of PIH. During the 5–10 h after a single injection of either chelator, the rate of biliary iron excretion sharply declines, but a second dose again stimulates the rate of ^{59}Fe excretion to the level seen after the first injection. With labeling of reticuloendothelial iron one hour before an injection of PIH, a different pattern of biliary excretion is seen. The first dose of PIH, given at a time when about 80% of the reticuloendothelial cell radioiron is in the form of hemoglobin that rapidly releases its iron into the plasma, results in a significant increase in ^{59}Fe excretion. By contrast, a second dose of PIH 12 h later, when almost all of the radioiron has been incorporated into reticuloendothelial cell ferritin, has a less pronounced effect. Thus, PIH is apparently able to chelate radioiron liberated from catabolized hemoglobin and excrete this iron through hepatocytes into the bile, but reticuloendothelial storage iron seems to be less available for chelation. In these studies in rats, repeated PIH *parenteral* administration was accompanied by an apparent decrease in liver and kidney radioactivity; but PIH did not increase biliary excretion of zinc or copper, although it can complex with both metals *in vitro* (see above). *Oral* administration of PIH after labeling of hepatic parenchymal iron also increased 24-h biliary, fecal, and urinary radioiron excretion. Interestingly, a significant increase in fecal excretion was found not only in rats with an intact biliary duct but also in animals whose bile was

collected using a biliary cannula, implying the existence of an extrabiliary pathway for the intestinal excretion of Fe-PIH_2. In other studies of PIH, 100 mg/kg, orally administered to rats, an increase in iron excretion of about 1.0 mg/kg/day was observed, corresponding to an apparent chelating efficiency of about 10%.[26]

The sites of iron chelation by PIH *in vivo* and the mechanism of excretion have also been examined by Hershko and colleagues in rats in which erythropoiesis had been suppressed by hypertransfusion.[27] Iron in hepatic parenchymal cells was selectively labeled with ^{59}Fe-ferritin, and that in reticuloendothelial cells with ^{59}Fe-loaded, heat-damaged red blood cells (^{59}Fe-DRBC). The effects on the urinary and fecal excretion of parenchymal iron (^{59}Fe-ferritin) and of reticuloendothelial iron (^{59}Fe-DRBC) produced by the administration of PIH by oral or intramuscular routes, and of the same amount of desferrioxamine (DF) intramuscularly were compared. After either oral or intramuscular administration of PIH, the excretion of hepatic parenchymal iron (^{59}Fe-ferritin) was confined entirely to the gastrointestinal tract. With reticuloendothelial iron (^{59}Fe-DRBC), most of the excretion was through the gastrointestinal tract, although a small but significant proportion of the iron was also excreted in the urine. The pattern of excretion of hepatocyte iron seen with desferrioxamine was similar to that of PIH; but with reticuloendothelial iron, a major portion of the chelated iron is excreted in the urine. For hepatic parenchymal iron, the cumulative excretion seen after oral administration of PIH was not significantly different from that observed after the same dose of desferrioxamine given intramuscularly (10.8 ± 1.3%). Thus, *PIH given orally was comparable in efficiency of iron chelation to parenteral desferrioxamine.* Dose-response studies showed that radioiron excretion increased in direct relation to the amount of PIH administered orally over a range of 125–500 mg/kg/day. Many of the results of these experiments have been reproduced in subsequent studies by these investigators.[14] Similar results have also been obtained using a screening assay for iron-chelators in which ^{59}Fe-ferritin is used to transiently label the main "physiologic" source of chelatable iron within the hepatocytes of normal rats.[28] After chelatable hepatocyte iron was labeled with ^{59}Fe-ferritin and intramuscular doses of PIH and desferrioxamine of equivalent iron-binding capacity were given, the maximum percentage of the labeled iron available to PIH for chelation (70%) was greater than the maximum available to desferrioxamine (40%). This result suggests that PIH, like desferrioxamine, probably acts primarily on hepatocyte pool(s) of transit iron rather than on storage iron. These results have since been confirmed in further studies.[29]

The studies by Hershko and co-workers[27] permit some further tentative inferences about the pharmacokinetic properties of PIH. With ^{59}Fe-ferritin labeling, almost 90% of the ^{59}Fe is localized to the liver and 97–100% of this liver iron is contained within hepatocytes. With this ^{59}Fe-ferritin hepatocyte label, PIH given orally or parenterally produces nearly identical fecal iron excretions, providing evidence for *almost complete gastrointestinal absorption of orally administered PIH.* By contrast, with ^{59}Fe-DRBC labeling, only about 40% of the ^{59}Fe-ferritin is contained within the liver (where 100% is found within reticuloendothelial cells), and the remaining ^{59}Fe is at extra-hepatic sites. With the ^{59}Fe-DRBC reticuloendothelial label, PIH given orally produced an iron excretion level over 40% *less* than that produced by PIH given parenterally. This observation implies that, at the dosage level used (about 200 mg PIH/kg), much of the PIH given orally was unable to reach and chelate iron at the extra-hepatic sites accessible to the parenterally administered drug. Given the evidence just cited for almost complete gastrointestinal absorption of orally administered PIH, this result suggests that, like other drugs such as propranolol, propoxyphene and lidocaine,[30] PIH is subject to considerable presystemic ("first-pass") hepatic elimination (i.e., it has a high hepatic extraction ratio).

Furthermore, Fe-PIH_2 iron chelate in the systemic circulation also undergoes hepatic elimination. After intravenous injection of ^{59}Fe-PIH_2, 98.5 ± 8.9% is excreted in the feces and only 1.5 ± 0.7% in the urine. Finally, the Fe-PIH_2 iron chelate, unlike ferrioxamine, can probably enter cells and donate iron back to them. Six days after intravenous administration of ^{59}Fe-PIH_2, some 32.6% of the ^{59}Fe remained within the experimental animals, while only 13.9% of the ^{59}Fe from ^{59}Fe-ferrioxamine had been retained.

The accumulated data from studies in animals suggest that PIH may be an iron chelator with a distinctive pharmacokinetic profile: a highly selective agent that (1) is almost completely absorbed from the gastrointestinal tract; (2) is delivered directly via the portal blood to the liver, where the drug has a high hepatic extraction ratio and intrinsic clearance; and (3) is taken up by the hepatocyte, its major site of action, where the drug either chelates iron and is excreted in the bile or is metabolized and eliminated. Excess iron at extra-hepatic sites may then be mobilized and transported by physiologic means to the liver for subsequent chelation and excretion. This mechanism of chelator action may serve to minimize the risks of systemic toxicity, because little of the active drug appears in the systemic circulation.

Safety of PIH

Safety of the Components, Pyridoxal and INH

Because of evidence that PIH may hydrolyze into its component compounds, pyridoxal and INH,[11] like other hydrazones of INH,[31] the safety of these components will first be considered. Pyridoxal is the form of vitamin B_6 with an aldehyde group in position 4 of the pyridine nucleus (pyridoxine has an alcohol and pyridoxamine, an aminoethyl group, in this position). All three forms of vitamin B_6 are converted in the body to pyridoxal phosphate; pyridoxal is converted to pyridoxal-5-phosphate by the enzyme pyridoxal kinase. In adults, the requirement for vitamin B_6 is estimated to be about 2 mg/day; the vitamin is found in meat, liver, whole grain breads and cereals, soybeans and vegetables.[32] Pyridoxal is excreted as 4-pyridoxic acid, formed by the action of hepatic aldehyde oxidase on free pyridoxal.[31a] Extremely large doses (2–6 g/kg) of vitamin B_6 produce convulsions and death in rats and mice,[32] but lower doses can be given daily without apparent effect. In humans, a toxic sensory neuropathy was originally described in individuals who consumed more than 2000 mg/day chronically,[33] but it may also occur in some individuals at lower doses.[34,35] Pyridoxal may interact with cycloserine, hydralazine, and levodopa; it should be avoided in patients receiving these compounds.[36] Isonicotinoyl hydrazide (isoniazid, INH) is still considered the primary drug for the chemotherapy of tuberculosis. The incidence of adverse reactions to INH was estimated to be 5.4% among more than 2000 patients treated with the drug, including rash (2%), fever (1.2%), jaundice (0.6%), and peripheral neuritis (0.2%).[37] Hypersensitivity reactions, hematologic abnormalities (agranulocytosis, eosinophilia, thrombocytopenia, anemia), vasculitis, arthritis and arthralgias, and central nervous system manifestations (convulsions, optic neuritis, and other disorders) have also been reported.[36] If pyridoxine is not given concurrently, peripheral neuritis is the most common reaction to INH, occurring in about 2% of patients. In the body, INH may combine with pyridoxal to form the hydrazone, PIH, and thereby inhibit pyridoxal kinase. Thus, INH appears to exert its anti-vitamin B_6 effect primarily by inhibiting the formation of the coenzyme form of the vitamin, pyridoxal-5-phosphate. Toxic effects of INH can be minimized by prophylactic therapy with pyridoxine (50 mg/day) and careful surveillance of the patient. In

some individuals, INH may cause severe hepatic injury leading to death.[38] The mechanisms responsible for this toxicity are unknown, but continuation of the drug after the development of hepatic dysfunction tends to increase the severity of the damage. Age seems to be the most important factor in determining the risk of hepatotoxicity with INH: hepatic damage is rare in patients less than 20 years old, is observed in 0.3% of those 20–34 years old, in 1.2% of those 35–49, and in 2.3% of those greater than 50 years of age.[38a] A much larger percentage of patients (up to 12%) may have elevated plasma transaminase activities. Most hepatitis begins 4–8 weeks after the start of therapy. INH must be given with great care to those with pre-existing liver disease.[39,40] INH is usually administered in doses of 5–10 mg/kg/day up to a maximum of 600 mg/day; the higher doses are used in more severely ill patients. INH diffuses into all body fluids and cells. From 75% to 95% of a dose of INH is excreted in the urine in 24 h, mostly as metabolites resulting from enzymatic acetylation and hydrolysis; small quantities of hydrazones and other derivatives are also detectable.[41]

Studies of the Safety of PIH In Vitro

PIH has been examined in cell culture systems with Chang cells, fibroblasts, and hepatocytes. Chang cells were incubated for 22 h in minimal essential medium with 50% serum, washed, and then incubated for 6 h with PIH (1 m*M*); no ill effects were reported.[15] PIH had no apparent effect on fibroblasts in culture.[19] Using cultures of hepatocytes from fetal (3–4-day-old cultures) and adult (2–3-day-old cultures) rats, Baker and colleagues[20] assessed the toxicity of PIH on the basis of morphological changes, trypan blue staining, and the release of aspartate aminotransferase (glutamic oxaloacetic transaminase, GOT) into the medium. No evidence of toxicity was observed with up to 24-h incubations in minimal essential medium with a 1 m*M* concentration of PIH.

Studies of the Safety of PIH In Vivo

Many of the studies of the effects of PIH in animals have been designed to determine only the physiologic effects of this agent; two published studies have also sought laboratory evidence of toxicity in animals treated with PIH. Hypertransfused adult female Wistar rats of the Hadassah strain were included in the studies of Hershko and associates.[27] PIH, 125–500 mg/kg/day, was given by gavage to 15 animals for 10 days without any evidence of toxicity; assessment included complete blood counts, renal function tests, and assays of serum proteins and liver enzymes. Subsequent studies of 12 normal rats given up to 200 mg PIH/kg by intravenous injection again found no abnormalities in complete blood counts, renal function tests, serum proteins and liver enzymes.[14] In our laboratory, no adverse effects of PIH were found in acute toxicity studies in mice or subacute (14-day) toxicity studies in rats and guinea pigs.[42,43]

PYRIDOXAL ISONICOTINOYL HYDRAZONE: CLINICAL STUDIES

Before reviewing the results of Phase I clinical trials of PIH, I will consider the question of what constitutes a clinically useful amount of chelate-induced iron

excretion. The body lacks any effective mechanism for the excretion of excess iron. Iron balance is physiologically regulated by controlling iron absorption: iron stores and iron absorption are reciprocally related so that absorption is reduced as stores increase. Normally, iron exchange is limited so that the adult male absorbs and loses only about 0.01 mg Fe/kg/day.[44] In patients with iron-loading anemias (such as thalassemia intermedia, hemoglobin E–β-thalassemia, sideroblastic anemia, and other disorders) who are not adequately transfused, gastrointestinal iron absorption may increase as much as tenfold, to 0.1 mg Fe/kg/day, as a result of an increased iron demand from the erythroid marrow due to ineffective erythropoiesis.[45] Most patients with Cooley's anemia require 200–300 ml/kg/yr of blood, an amount equivalent to 0.25–0.40 mg Fe/kg/day.[46] Thus, in untransfused patients with iron-loading anemias, a chelate-induced iron excretion of only 0.05 mg Fe/kg/day could considerably retard the accumulation of a lethal body iron burden. An excretion of 0.1 mg Fe/kg/day could stop further iron loading, and any greater excretion would produce a negative iron balance. In transfusion-dependent patients, a chelate-induced excretion of 0.2 mg Fe/kg/day, by slowing the rate of iron loading, would improve survival. Excretion equivalent to the transfusional iron load would be achieved at 0.25–0.4 mg Fe/kg/day; higher levels of excretion would progressively decrease the body iron burden.

Studies of the safety and effectiveness of PIH in healthy volunteers and patients with iron overload were carried out in our laboratory with the approval of the Committee on Human Investigation at Cleveland Metropolitan General Hospital under the provisions of a physician-sponsored Claimed Investigational Exemption for a New Drug, for PIH, from the Food and Drug Administration (FDA IND No. 28545). The results of these studies have been presented in brief form elsewhere.[42,43] Initial Phase I dosage studies were carried out in five healthy volunteers given escalating daily oral doses of about 2–30 mg PIH/kg/day for two weeks without ill effect. Additional Phase I dosage studies were then carried out in 11 volunteers with iron overload due to transfusion or iron-loading anemia.[42] To minimize the risk of drug toxicity, a low initial dose of PIH was deliberately chosen (about 30 mg PIH/kg). In a randomized, double-blind manner, these volunteers were given PIH or placebo every 8 h for 6 days while receiving a constant, low-iron diet (6–10 mg Fe/day). The 6-day treatment intervals were preceded and followed by 3-day equilibration periods when no drug was given. Ascorbate was not given. PIH or placebo were given 30 min after calcium carbonate, 1800 mg, in an attempt to minimize acid hydrolysis of PIH in the stomach, and at least 2 h before meals to avoid chelation of dietary iron by PIH. Non-invasive magnetic measurements of hepatic magnetic susceptibility were used to determine liver iron concentrations.[47] But for one volunteer with mild transient nausea, reports of side effects were similar with PIH and placebo. Hepatic non-heme iron concentrations, determined by magnetic susceptibility, were 2296 to 15,986 μg Fe/g liver (wet weight; normal is about 50 to 500 μg Fe/g). Serum ferritin concentrations ranged from 381 to 12,735 μg/l[42]. No ophthalmologic or audiologic abnormalities developed in any of the volunteers. Hematologic, serum, plasma, and urine biochemical measurements (including complete blood count, transferrin saturation, serum ferritin, alkaline phosphatase, aspartate and alanine aminotransferases, bilirubin, lactate dehydrogenase, creatinine, plasma and urine zinc) were similar with PIH and placebo. Iron excretion was determined as the difference in the amount of iron in the stool and urine during periods of treatment with PIH and with placebo. Iron balance could not be evaluated in 2 volunteers. In the remaining 9 volunteers, the mean total iron excretion with an average daily dose of about 30 mg PIH/kg was 0.12 ± 0.07 mg Fe/kg/day (range, 0.04 to 0.22 mg Fe/kg/day). The mean overall apparent efficiency of chelation was 4.1 ± 2.6% (range, 1.1–8.1%), based on a maximum of 176 mg of iron that could be chelated by the 1800 mg daily dose of PIH.

As noted above, a low initial dose of PIH (about 30 mg PIH/kg) was deliberately chosen to minimize the risk of drug toxicity in these initial trials. A daily iron excretion within the range achieved in this study with even this low dose of PIH (0.12 ± 0.07 mg Fe/kg/day) would be helpful in delaying or preventing iron accumulation in patients with iron-loading anemias who are not regularly transfused. Transfusion-dependent patients with Cooley's anemia would need a still greater daily excretion, of 0.25–0.40 mg Fe/kg, to keep pace with the transfusional iron load. While the dose of PIH administered could simply be increased, the known toxicity of the constituents of this agent, pyridoxal and isoniazid, suggests the need for caution. Moreover, the mean overall apparent efficiency of chelation by PIH was only 4.1 ± 2.6% (range, 1.1–8.1%). For further clinical studies, we have chosen an alternative approach to improve the bioavailability of PIH and thereby enhance iron excretion without increasing the risk of toxicity. An enteric-coated preparation to protect the PIH until the chelator reaches the small intestine is being examined. In addition, the usefulness of the co-administration of pyridoxal is being evaluated as a means of enhancing the bioavailability of PIH and increasing iron excretion. Co-administration of pyridoxal may enhance the delivery of PIH to the liver (1) by diminishing spontaneous dissociation of PIH into its components, pyridoxal and isoniazid, by shifting the equilibrium in the dissociation reaction toward PIH; and (2) by competitively inhibiting Schiff-base reactions which result in the transfer of the pyridoxal moiety of PIH to the ϵ-amino group of lysyl and other amino acid residues in proteins. While no data from studies of the dissociation of PIH under physiologic conditions are available, the closely related compound pyridoxal phosphate isonicotinoyl hydrazone (i.e., the hydrazone formed between pyridoxal phosphate and INH) readily undergoes spontaneous dissociation in aqueous solution.[11] Increasing the concentration of pyridoxal would be expected to shift the equilibrium in the dissociation reaction toward PIH. Pyridoxal readily binds to serum albumin ($K_a = 340\ M^{-1}$),[31,48,49] and it seems likely that the pyridoxal moiety of PIH is lost in Schiff-base reactions with the ϵ-amino group of lysyl and other amino acid residues in albumin and other proteins.[11,31] Increasing the concentration of pyridoxal would be expected to competitively inhibit these Schiff-base reactions. It is postulated that co-administration of pyridoxal will produce an increase in the pyridoxal concentration in the intestinal lumen, in portal blood, and within the hepatocyte, thereby increasing the amounts of PIH reaching sites of iron chelation. Co-administration of pyridoxal is thus a potential means of increasing the bioavailability of PIH and improving the efficiency of iron chelation without increasing the amounts of INH to which patients will be exposed.

CONCLUSION

A review of the preclinical information that has been amassed by a number of investigators and of results from our own laboratory shows that PIH is a compound of great promise for the treatment of iron overload. Our Phase I clinical trial has shown that PIH can safely produce a level of iron excretion that would be clinically useful in the treatment of non-transfusion-dependent patients with iron-loading anemias. While a higher level of iron excretion would be needed for the management of transfusion-dependent patients, these initial results suggest that a more bioavailable formulation of PIH should be evaluated as a means of increasing iron excretion before increasing the dose of the chelator.

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The Development of Hydroxypyridin-4-ones as Orally Active Iron Chelators

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CONCEPTS LEADING TO THE DESIGN OF 3-HYDROXYPYRIDIN-4-ONES FOR THE SELECTIVE REMOVAL OF IRON

The obvious method of choice for the design of iron chelators is to model novel structures on natural siderophores, which possess extremely high affinities for iron(III).[1] These structures are typically based on the hydroxamate and catechol moieties. Unfortunately, hydroxamates are susceptible to the acid environment of the stomach, and some are subject to enzyme-catalyzed cleavage. Consequently, they possess low oral activity. Although the catechol function is more stable, most natural catechol-containing siderophores are still hydrolyzed in the intestinal tract. In an attempt to overcome this difficulty, several research groups have designed non-hydrolyzable analogues of the natural siderophore enterobactin.[2] Like enterobactin, these molecules possess an extremely high affinity for iron(III) but are insoluble in water. Although this limitation can be avoided by the introduction of sulphonic acid functions, the resulting water-soluble derivatives are poorly absorbed. A further disadvantage associated with catechol siderophores is that they form highly charged iron(III) complexes which tend to trap iron in intracellular compartments. In addition to these problems many siderophore analogues are capable of donating iron to pathogenic organisms, and consequently neither hexadentate catechols or hydroxamates appear to be well suited to the task of scavenging and removing iron from iron-overloaded mammals.

In order to design molecules which possess a high selectivity for iron(III), and yet lack the disadvantages of the catechol and hydroxamate moieties, a search was made for monoprotic bidentate iron(III) ligands. There is a range of such aromatic nuclei which are capable both of co-ordinating iron(III) with high affinity and of forming neutral complexes. These include tropolone, 8-hydroxyquinoline, hydroxypyran-4-ones, hydroxypyridin-2-ones, and hydroxypyridin-4-ones. A systematic analysis of the

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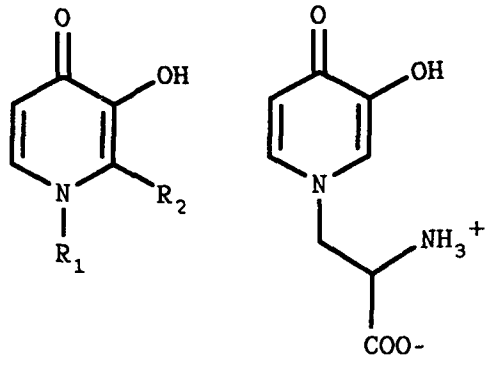

3-Hydroxypyridin-4-one Mimosine

FIGURE 1. Structures of 3-hydroxypyridin-4-one (*left*) and mimosine (*right*).

properties of these ligands identified 3-hydroxypyridin-4-ones (FIG. 1) as possessing optimal properties for clinically useful iron chelators.[3,4] These compounds possess a high affinity for iron(III) ($\beta_3 = 10^{36}$) and form a neutral 3:1 complex under most physiological conditions (FIG. 2). Indeed, the 3-hydroxypyridin-4-ones may be considered as hybrid structures containing elements of both catechol and hydroxamate (FIG. 3).

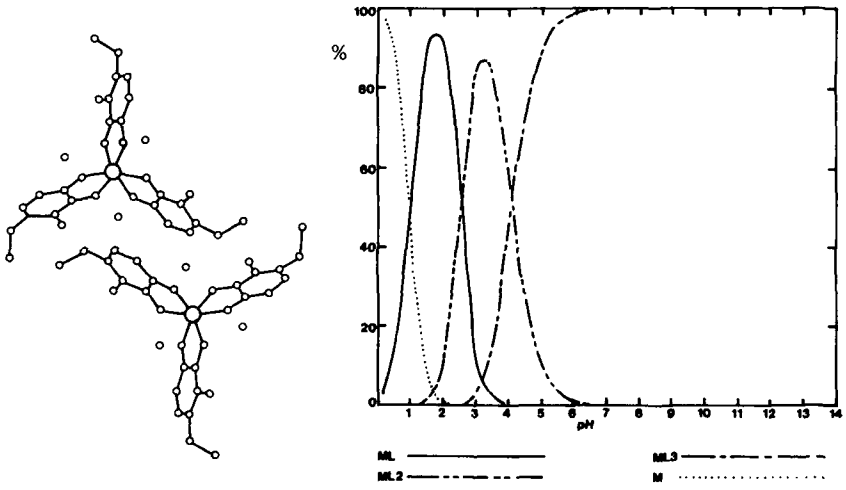

FIGURE 2. The crystal structure (*left*) of the iron(III) complex of 2-ethyl-3-hydroxy-1-methylpyridin-4-one (CP21). (Diagram courtesy of R. van der Helm and G. Xiao, University of Oklahoma.) Speciation plot (*right*) of 3-hydroxypyridin-4-one and iron(III). Concentration of ligand (L), 4×10^{-4} *M*; concentration of iron(III) (M), 1×10^{-4} *M*. (From Hider & Hall.[4] Reprinted with permission from *Progress in Medicinal Chemistry*.)

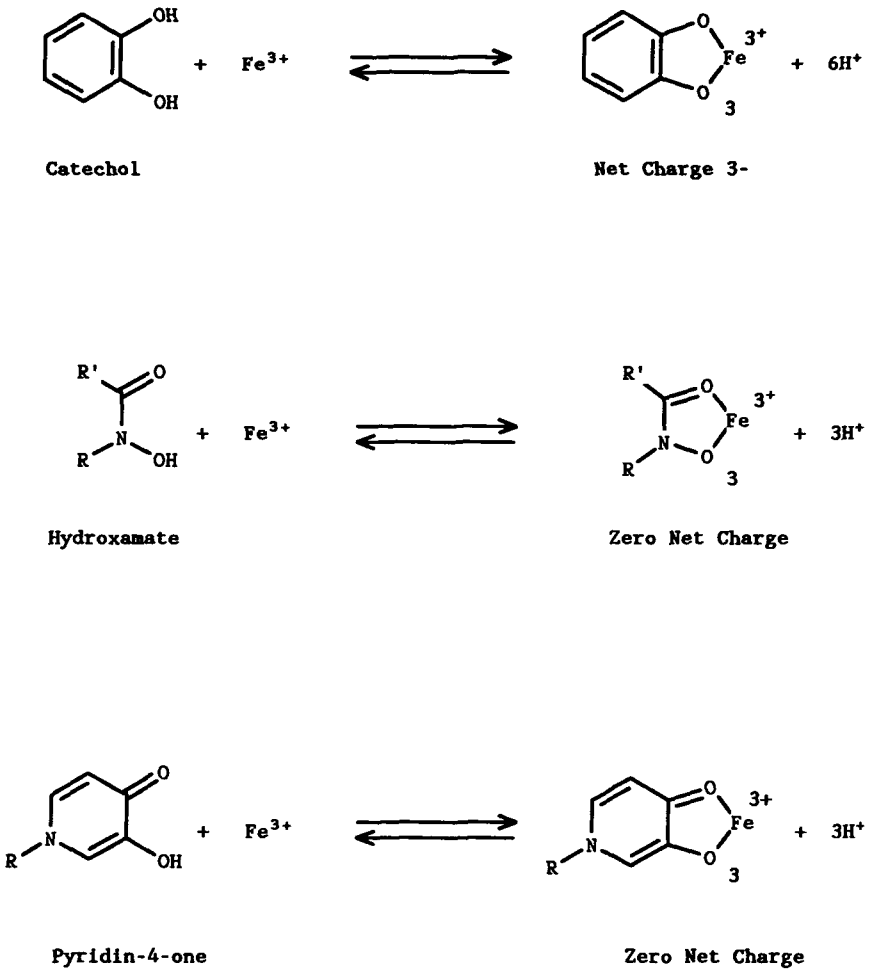

FIGURE 3. Comparison of the chelation of iron(III) by catechol, hydroxamate, and pyridin-4-one ligands. Each ligand forms a 3:1 complex with iron(III). Both the hydroxamate and pyridin-4-one complexes are neutral as only a single proton is displaced from each ligand on chelation. In contrast, the catechol complex is highly charged due to the displacement of 2 protons from each ligand on chelation.

DESIGN AND SYNTHESIS OF 3-HYDROXYPYRIDIN-4-ONES

3-Hydroxypyridin-4-ones possess a structure similar to that of phenol and catechol and therefore might be expected to bind to, and possibly inhibit, enzymes catalyzing reactions based on these groups, such as catechol-*O*-methyltransferase, aromatic amino acid hydroxylase, and tyrosinase. Indeed, mimosine (FIG. 1) has been demonstrated to inhibit these enzymes, and consequently it is toxic to mammals. However, the introduction of an alkyl function on ring position 2 (R_2) of

3-hydroxypyridin-4-one (FIG. 1) markedly reduces the inhibitory properties of this class of molecule, without adversely influencing the affinity for iron(III).[5] The octanol/water partition coefficient (K_{part}) of both the ligand and the neutral iron(III) complex can be controlled by varying the size of the alkyl substituents on both positions 1 and 2 (R_1 and R_2) of the 3-hydroxypyridin-4-one ring (FIG. 1).

The basic method of 3-hydroxypyridin-4-one synthesis was first reported in 1932[6] and has subsequently been modified by many workers.[7-11] Kontoghiorghes and Sheppard have recently reported this well-established synthetic route to be novel.[12]

COMPETITION STUDIES WITH IRON-PROTEIN

The 3-hydroxypyridin-4-ones do not remove iron from heme proteins such as hemoglobin, cytochrome c, and cytochrome P_{450}. In contrast, they are capable of removing iron from both ferritin[13] and hemosiderin[14] under physiological conditions. The pM value[15] for 3-hydroxypyridin-4-one is lower than that of transferrin (TABLE 1); therefore, when present at low concentrations (50 μM), pyridin-4-ones do not remove iron from transferrin efficiently. Indeed, as might be predicted from this relative pM value, the iron complexes of the pyridin-4-ones rapidly donate iron to apotransferrin.[16]

TRANSMEMBRANE MOVEMENT OF 3-HYDROXYPYRIDIN-4-ONES AND THEIR IRON(III) COMPLEXES

The membrane permeability of small solutes (molecular weight < 400) is dominated by the ability of the solute to partition from an aqueous phase into the low dielectric of the membrane environment. A good estimate of this partitioning phenomenon can be obtained from the octanol/water (pH 7.4) partition coefficient (K_{part}). The K_{part} values of some 3-hydroxypyridin-4-ones and of their corresponding iron(III) complexes are presented in TABLE 2. With the exception of CP24, the values are higher for the free ligand than for the iron(III) complex. Indeed, with the short chain substituents (methyl and ethyl), there is often an order of magnitude difference between the values for the free ligand and for the complex. With the longer side chains, as in CP22 (*n*-propyl), CP24 (*n*-butyl), and CP52, the difference is less marked.

Studies with liposomes have demonstrated that 3-hydroxypyridin-4-ones rapidly penetrate lipid bilayer membranes. Thus, the distribution of the noncharged pyridin-4-ones, CP21 and CP94, reach equilibrium within 2–3 min. In contrast, charged pyridin-4-ones (e.g., CP44) behave like desferrioxamine and only penetrate lipo-

TABLE 1. pM Values of Selected Ligands with Iron(III)

Ligand	pM[a]
3-Hydroxypyridin-4-one	20.0
EDTA	22.2
Apotransferrin	23.6
Desferrioxamine	26.6
Enterobactin	35.5

[a]pM values offer a means of directly comparing the affinity of different ligands for iron(III) at pH 7.4.[15]

TABLE 2. Partition Coefficients of 3-Hydroxypyridin-4-ones

Compound	R_1	R_2	Partition Coefficient (K_{part}) Free Ligand	Iron (III) Complex
CP20	CH_3	CH_3	0.21	0.0009
CP21	CH_2CH_3	CH_3	0.4	0.03
CP22	$(CH_2)_2CH_3$	CH_3	1.35	0.65
CP23	$CH(CH_3)_2$	CH_3	1.45	0.20
CP24	$(CH_2)_3CH_3$	CH_3	1.98	7.7
CP25	$(CH_2)_4CH_3$	CH_3	>20	>20
CP40	$(CH_2)_2OH$	CH_3	<0.002	<0.002
CP44	$(CH_2)_2NH_2$	CH_3	<0.002	<0.002
CP51	$(CH_2)_2OCH_3$	CH_3	0.3	0.005
CP54	$CH(CH_3)CH_2OCH_3$	CH_3	0.6	0.03
CP52	$(CH_2)_3OCH_2CH_3$	CH_3	1.0	0.39
CP93	CH_3	CH_2CH_3	0.5	0.03
CP94	CH_2CH_3	CH_2CH_3	0.85	0.07
CP96	CH_2CH_2OMe	CH_2CH_3	0.83	0.046

somes slowly. As would be expected from their more hydrophilic nature and much higher molecular weight, the iron(III) complexes of hydroxypyridinones permeate liposome membranes more slowly than do the corresponding free ligands (unpublished observations).

3-HYDROXYPYRIDIN-4-ONE–FACILITATED REMOVAL OF IRON FROM HEPATOCYTES

As a large proportion of iron in iron-overloaded animals (including man) is present in the liver, hepatocytes have been developed as an *in vitro* assay for the identification of clinically useful chelators.[17] The pyridinone chelators are highly efficient at removing intracellular iron in this system; indeed, many are more effective than is desferrioxamine. A range of pyridin-4-ones was investigated in this model, and the ability of these compounds to remove iron from hepatocytes correlated well with the K_{part} values of the free ligands ($R = 0.94$).[17] A K_{part} value in the range 0.3–1.0 was found to be ideal for the maximal effect; values above this range were associated with cell damage.

COMPARATIVE IRON MOBILIZATION IN ANIMAL STUDIES

After the compounds which possess the optimal properties for both maximum iron mobilization and minimum toxicity *in vitro* were identified, a small number of hydroxypyridin-4-one chelators were evaluated in animals. The model chosen was relatively simple, one using mice first overloaded with iron dextran and then given trace labeling with ^{59}Fe-lactoferrin. Following a re-equilibration period of at least two weeks, the majority of the radioiron is found in the liver, as assessed by gamma-counting of the various organs, and specifically in hepatocytes, as shown by autoradiography (unpublished observations). Initial findings confirmed that there was a broad agreement between iron mobilization from hepatocytes in tissue culture

and the ability of different compounds to increase iron excretion by both the oral and intraperitoneal routes.[18,19]

More extensive studies using the mouse model have shown that a number of hydroxypyridin-4-one chelators with K_{part} values between 0.3 and 1 are highly effective by the oral route at producing increased fecal and urinary excretion,[18,20] the majority of iron excretion being in the feces. Of these, the 2-methyl derivatives CP51, CP21 and CP22 and the 2-ethyl derivatives CP93, CP94, and CP96 are all significantly more effective in these short-term dose-response experiments than is CP20 (L1). In FIGURE 4, the relative effectiveness of CP94 is compared with that of CP20 at four dose levels. CP94 is significantly more effective than CP20, in contrast to the recent

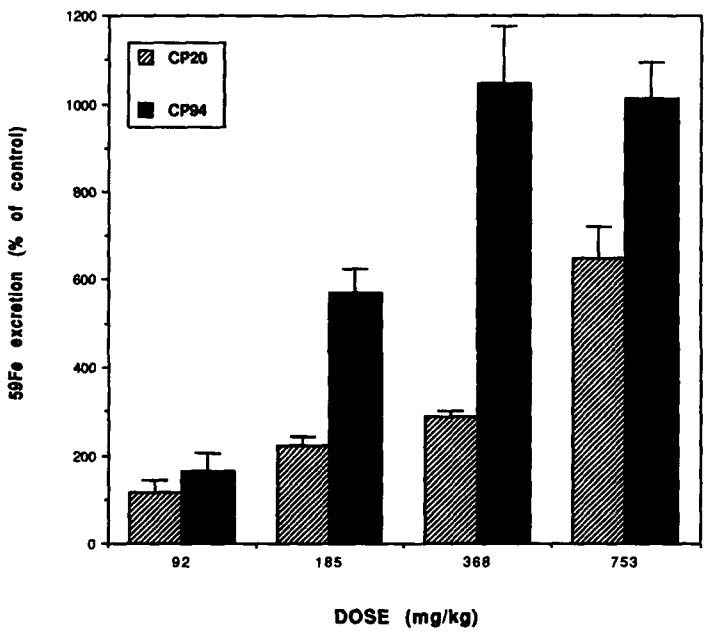

FIGURE 4. Total ^{59}Fe excretion (urine + feces) for iron-overloaded mice expressed as a % of baseline excretion for each mouse is compared for ascending oral doses of CP20 and CP94. Values shown are the mean and standard error obtained with six mice for each chelator.

claims by Kontoghiorges and Hoffbrand.[21] Recent work by HERSHKO ET AL. (this volume) and by Pippard (personal communication) also show CP94 (as well as other compounds) to be markedly more effective than CP20.

Although these single-dose studies are a convenient way of comparing the efficacy of several compounds, it is pertinent to consider whether these chelators can significantly reduce iron overload when given for a long period of time. In a two-month study, five hydroxypyridin-4-one chelators (CP20, CP21, CP94, CP93, CP51) were selected for comparison with desferrioxamine (DFO).[22] A total of 78 mice were randomly divided into two groups. Half of these were iron overloaded by weekly intraperitoneal injections of iron dextran over four weeks (total, 8 mg of

iron). Following a two-week equilibration period, mice received test chelators at a dose of 200 mg/kg daily for 60 days by the intraperitoneal route. Animals were then sacrificed, and the non-heme iron content in liver, spleen, and heart was determined. The distribution of tissue iron was also examined in these organs histologically using Perl's stain. With the exception of CP20, the hydroxypyridin-4-ones caused a significant reduction (>50%) in liver iron in the overloaded mice ($p = 0.003$) which was greater than that observed with DFO (42%). The relative activities of the hydroxypyridiones agreed well those seen in cultured hepatocytes and in short-term animal models with the oral route of administration. CP51 (63% reduction) was the most effective and CP20 (L1) the least effective (38% reduction). All compounds were inefficient at removing iron from the reticuloendothelial system as compared to removal of parenchymal iron. Only CP51 produced a significant reduction in splenic iron ($p = 0.003$). These results confirm that the most effective compounds at causing excretion of radioiron in the acute mouse model were also the most effective at removing significant amounts of iron in longer-term studies.

COMPARATIVE TOXICITY STUDIES IN ANIMALS

With the emergence of candidate iron chelators over the past two decades, many compounds have been shown to be effective at causing iron excretion in animal models, only to be shown to have unacceptable toxicity at a later stage of their evaluation. (TABLE 3). It is therefore important to perform detailed evaluation of toxicity-efficacy relationships before proceeding to clinical studies. Experience from the use of DFO clinically has shown that the toxic effects of this drug are particularly marked in non–iron-overloaded patients. Therefore, the screening of chelators in non-overloaded animals, whilst highlighting toxicities directly related to iron chelation, may overestimate the potential toxicity of the chelators in iron-overloaded patients. For this reason, we have performed much of our toxicology studies in both iron-loaded as well as non-overloaded animals. Simple LD_{50} studies have shown a close dependence of acute toxicity on lipid solubility. There is a sharp rise in acute toxicity when the K_{part} value exceeds approximately 1, while below this value acute toxicity is relatively independent of K_{part} values.[22] By contrast, oral efficacy increases between K_{part} values of 0.2 and 1. Thus, compounds with intermediate lipid solubility—with K_{part} values close to but somewhat below 1—possess the greatest therapeutic safety margin in short-term studies. By relating the LD_{50} value to the dose of each chelator required to increase iron excretion by a given amount, an acute therapeutic safety index was obtained for each compound. CP94 has the best index in these studies.[19,22]

Details of longer-term animal toxicity with hydroxypyridin-4-ones have unfortunately been lacking until recently. A two-month study with iron-overloaded and non-overloaded mice, referred to above, was designed to compare the toxicity and efficacy of several of the most active hydroxypyridin-4-ones and to compare these with DFO.[23] Biochemical test data, hematological blood analysis, and histological tissue sections were obtained on control and treated animals at the end of 60 days. The results showed no significant abnormality of serum biochemistry and no histological abnormality of spleen, heart, lungs, joints, or eyes with any compound. All the 3-hydroxypyridin-4-ones studied were associated with centrilobular accumulations of eosinophilic material without evidence of associated necrosis or inflammatory infiltrate. This varied from being just detectable with some compounds to being more overt with others. The hematological abnormalities of leucopenia and anemia found

TABLE 3. Orally Active Iron Chelating Compounds: Current Status

Group/Compound[a]	Advantages	Disadvantages[b]	Stage of Development
Hydroxamate			
Cholylhydroxamic acid	Acceptable acute & subacute toxicity	Diarrhea in clinical studies at 100 mg/kg/day	None further
Prodrug of DFO	Presumed low toxicity	Insufficient activity in animals	More active derivatives awaited
Catecholates			
2,3-Dihydroxybenzoic acid	Acceptable acute toxicity	Poor iron excretion in humans at 100 mg/kg/day	None further
MECAM	High affinity for iron(III)	Septicemia in animals	None further
Aminocarboxylates			
Esters of HBED	Very active in animal models	Appreciable affinity for zinc	Animal subacute toxicity data awaited
Orthosubstituted phenolates			
PIH	Low toxicity in animals & humans	Poor activity in humans at 30 mg/kg/day	Search for more active analogues
Desferrithiocin	High oral activity in rats & dogs	Unacceptable subacute toxicity	None further by Ciba-Geigy
Hydroxypyridin-4-ones			
CP20 (L1)	Orally active in animals & humans	Barbiturate interaction, hypersalivation & ERG changes in rats Decrease in Hb & WCC in subacute studies in mice No fall in serum ferritin at 1 yr; little fecal iron excretion in humans	Formal subacute toxicity, pharmacokinetics & mutagenicity testing awaited Pilot studies in humans continue
CP94	More iron excretion in animal models than with CP20 Lacks certain toxicity observed with CP20 Not mutagenic	Not yet introduced in humans	Formal subacute toxicity in two species & pharmacokinetics in progress Pilot studies in humans imminent

[a]DFO, desferrioxamine; MECAM, 1,3,5-*N,N',N''*-tris(2,3-dihydroxybenzoyl) triaminomethylbenzene ; HBED, *N,N'*-bis(2-hydroxybenzoyl) ethylenediamine-*N,N'*-diacetic acid; PIH, pyridoxal isonicotinoyl hydrazone.
[b]ERG, electroretinograph; Hb, hemoglobin; WCC, white blood cell count.

TABLE 4. The Influence of CP20 on Hemoglobin Concentration and White Blood Cell Count

Treatment[a]	Hemoglobin (g/dl)	White Blood Cells (10^9/l)
Control	15.5 ± 0.2	7.7 ± 1.1
CP20 (L1)	13.6 ± 0.5	3.0 ± 0.4

[a] $n = 5$ for each.

with CP20 (L1; TABLE 4)[24] were not seen with other hydroxypyridinones. Deaths before the end of the experiment ranged from 0% (DFO and CP21) to 50% (CP20, [L1]) in the non-overloaded animals. By contrast, no deaths were observed in the overloaded animals treated with any of the chelators, again suggesting that iron overload protects against the toxic effects of high doses of chelators, in agreement with previous short-term results.

There are a number of other considerations in respect to the toxicity of these chelators. A number of independent groups (personal communications, P. Good and R. J. Bergeron; our own unpublished observations) have noted that rats anesthetized with barbiturates have prolonged anesthesia if exposed to CP20. In contrast, this has not been observed with compounds such as CP21 and CP51. Additionally, hypersalivation and sweating, which has not been observed with hydroxypyridin-4-ones possessing larger substituents in the 1 position on the pyridinone ring, has been observed in rats given CP20 (L1). These differences may relate to differences between the metabolism of L1 and the other hydroxypyridin-4-ones (see below). Electroretinographic changes similar to those induced by DFO have been observed in rats treated with CP20 (L1) by P. Good, D. Blake and co-workers (personal communication). These changes were absent with CP51.

From the results of both the acute and subacute studies on toxicity-efficacy relationships, as well as from the special toxicity studies, CP94 is the compound which appears to have the best balance between oral efficacy and toxicity.[22,23] We have therefore chosen to take this compound into tests on humans with the specific aim of establishing whether negative iron balance can be achieved in short-term studies at doses which are non-toxic.

TABLE 5. U.K. Department of Health (DH) Requirements for 7-Day Study of a New Drug in Humans

Stability, purity and formulation of compound
General pharmacology
Mutagenicity; Ames testing
Pharmacokinetic information in rats
Likely effects on cardiorespiratory system (acute)
Acute toxicity (LD_{50})
Full toxicology to GLP (good laboratory practice) standards:
28 days oral administration, 2 species (1 non-rodent)
3 doses (top deliberately toxic, bottom therapeutic)
10 males and 10 females at each dose level
Clinical observations, weight gain
Blood chemistry, hematology
Opthalmology, urinalysis
Full histology and necropsy

FURTHER REQUIREMENTS BEFORE CLINICAL TRIALS COMMENCE

We intend to satisfy the regulatory requirements both in the United Kingdom and in the United States for toxicology, pharmacokinetics, pharmacology, pharmaceutical purity, and stability before proceeding with clinical trials. These requirements are extensively described in the appropriate governmental guidelines and have been summarized.[25] TABLE 5 lists the basic requirements. The period and extent of

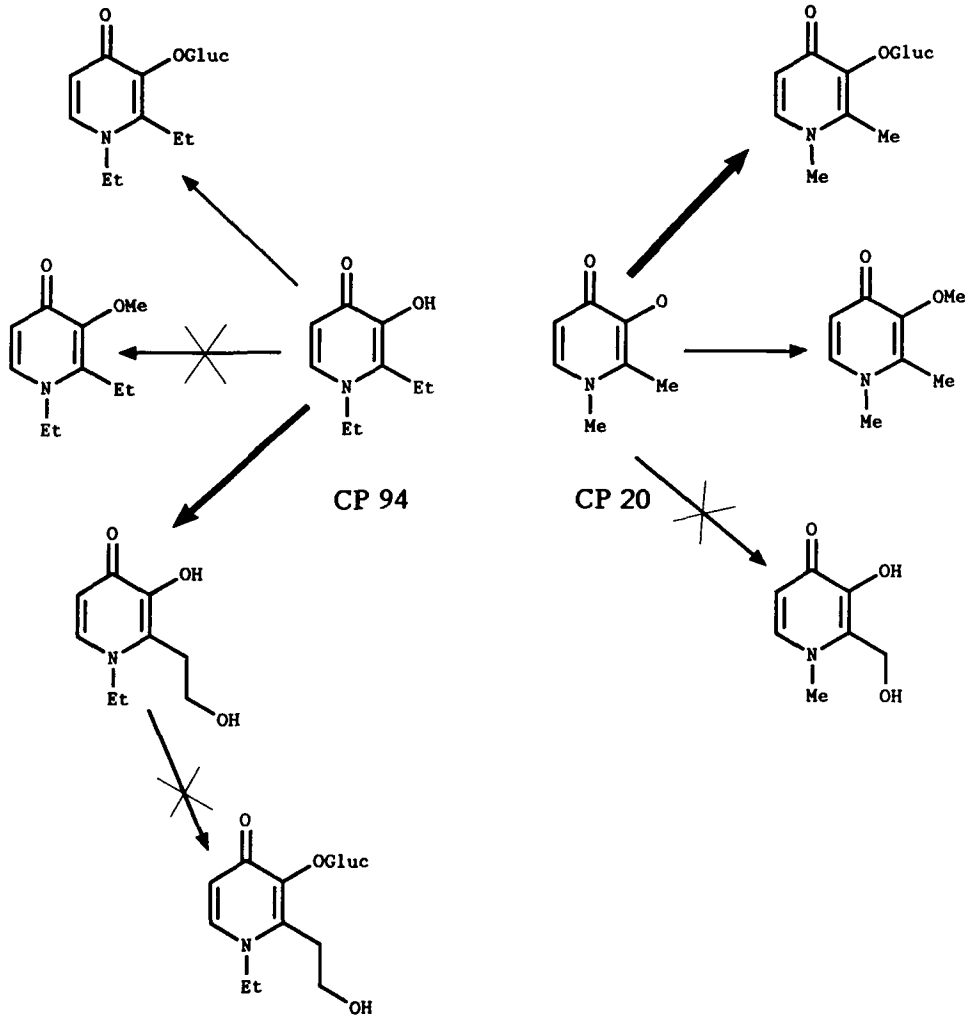

FIGURE 5. Comparisons of the metabolic routes of CP20 (L1) and CP94. The major metabolite of CP20 is the non-chelating glucuronide, whereas with CP94 it is a hydrophilic pyridin-4-one, which is itself capable of chelating iron(III). Arrows which are marked (×) indicate that there was no detectable metabolism by the route shown.

toxicological assessment will vary in proportion with the duration of the intended clinical trial. Our intention is to commence clinical trials with a dose-ranging study measuring urinary iron alone, followed by a formal metabolic balance study comparing CP94 with DFO. The metabolic balance protocol is crucial if meaningful results on fecal iron excretion are to be obtained. Previously reported studies with CP20[26,27] appear not to have been performed with sufficient run-up or run-off time to determine fecal iron excretion adequately.

For the other requirements (TABLE 5), much information has already been obtained. The chemical purity and stability of CP94 has been demonstrated by HPLC, ^{1}H-NMR, ^{13}C-NMR, mass spectroscopy, and elemental analysis. The Ames test for both metabolized and unmetabolized CP94 is negative. Preliminary pharmacokinetic and metabolism data have been obtained of CP94. There is a marked difference in the major metabolic routes for CP94 and CP20. Whereas the dominant route for CP20 (L1) is glucuronidation to form a non-chelating metabolite (FIG. 5), the dominant route for CP94 is to a more hydrophilic hydroxylated derivative which retains the ability to chelate iron(III).

Detailed formal toxicology testing in non-overloaded rats, including biochemistry, hematology, necropsy and histopathological examination, has been performed at three dose levels with CP94 given for one month intragastrically to good laboratory practice (GLP) guidelines. Toxicity testing in a second (non-rodent) species is now awaited. As with CP20 (L1),[28] macroscopic adrenal hyperplasia is noted at high doses; and moderate increments in serum cholesterol, but still within the normal range, have been noted at intended therapeutic doses. No change in white blood cell count has been noted at any dose level. A moderate reduction in hemoglobin values has been noted at 100 mg/kg.

CONCLUSIONS

From these systematic studies, CP94 has emerged as our present lead compound. We have commenced the formal toxicological and pharmacological investigations before introducing this compound into humans. On the basis of the information available, CP94 is predicted to be both more effective and less toxic than L1. It is our intention to perform detailed metabolic balance studies with CP94 in iron-overloaded volunteers, pending the results of the toxicity studies in a second animal species.

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Design, Properties, and Effective Use of the Oral Chelator L1 and Other α-Ketohydroxypyridines in the Treatment of Transfusional Iron Overload in Thalassemia[a]

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INTRODUCTION

The treatment of transfusional iron overload in β-thalassaemia and other diseases using desferrioxamine (DF: Desferal; Ciba-Geigy) is limited to a very small number of patients worldwide because of its high cost and oral inactivity. In India for example, of the 5000 patients born each year, only 5% may afford treatment with DF; and in Cyprus provision of DF to all patients under the national health scheme accounts for 40% of the national budget for drugs.[1] In developed countries where DF is available many patients do not comply with the 8–10 h/day subcutaneous administration of the drug; this non-compliance has fatal consequences. When DF is used effectively it can prolong the life of β-thalassaemia patients,[2] and some have reached the age of 40 years. Despite the high safety margin of DF, many toxic side effects have been noted, especially at high doses (over 50 mg/kg) and in patients with low iron stores. These include ocular and auditory abnormalities, cerebral toxicity, growth failure and bone abnormalities, Yersiniasis, etc. Many chelators have been tested in animals and few in humans for replacing DF with an oral, cheap, iron chelator. Of these, only one, namely 1,2-dimethyl-3-hydroxypyrid-4-one (L1), has been shown to be effective in iron removal in humans[3–5] and is undergoing multicenter clinical evaluation in nine countries, involving 130 patients (TABLE 1).

THE DESIGN OF L1 AND OTHER α-KETOHYDROXYPYRIDINES

The search for an alternative chelator to DF is based on three main criteria: oral activity, low cost, and low toxicity. All the chelators tested previously which were based on catechol and phenol groups, hydroxamic acids, polyaminocarboxylic acids, pyridoxal isonicotinoyl hydrazones, etc., had major drawbacks in their use.[6–7] An original search for the design of new chelators began in 1978 when J. Silver of Essex University suggested that a known commercial chelator, namely, 2-hydroxypyridine-

[a]This work was supported by the U.K. Thalassaemia Society.

TABLE 1. Multicenter Clinical Trials with L1

Location	Patients			Trial Period (months)	Single dose (mg/kg/day)
	Diagnosis	*n*	Ages (yr)		
U.K./London	β-Thalassemia	9	12-38	1-15	20-100
	Myelodysplasia	8	51-83	0.2-13	
	Renal dialysis	11	39-70	1 day	
	Other	7	28-63	1-5.5	
India/Bombay	β-Thalassemia	34	8-22	2-6	25-75
Switzerland/Berne	β-Thalassemia	8	11-27	4-9	55-80
Italy/Milan	β-Thalassemia	8	10-23	1.0	20-50
Italy/Messina	β-Thalassemia, sickle cell anemia	6	11-32	6-8	42-74
Netherlands/ Amsterdam	HbE- β-Thalassemia, myelodysplasia, aplastic anemia	4	15-85	2-3	20-70
Netherlands/ Rotterdam	Rheumatoid arthritis	10	56-83	0.2-0.8	11-25
Canada/Toronto	β-Thalassemia	20	15-27	0.2-1.0	17-25
Belgium, Greece, U.S.A.	β-Thalassemia, myelodysplasia	5	16-60	0.5-3.0	20-50

1-oxide (L4), which resembles an aromatic hydroxamate,[8,9,9a] might be suitable for replacing DF. However, the results of *in vitro* screening were dissappointing because L4 was charged and formed precipitates with iron at pH 7.4.[8] Further dissappointments were on the way. An attempt was made to increase the water solubility of the iron complex by introducing nitro substituents. This resulted, however, in a decrease in the affinity for iron due to the electron-withdrawing effects of the nitro group(s) and also in a drop of the pK_a of the hydroxyl group.[8,9] Investigation of the literature at that time revealed that other heteroaromatic chelators with an α-ketohydroxy iron-binding site (not hydroxamate), such as tropolone[10,11] and mimosine,[12-14] had high specificity for iron at physiological pH; they were absorbed orally and increased iron excretion following oral administration.[11,14] Absorption following oral administration was also reported for two other groups of α-ketohydroxy heteraromatic iron chelators, namely, maltol/ethyl maltol[15] and omadine.[16] On the basis of these observations, the search for simple methods of synthesis of α-ketohydroxy heteroaromatic chelators from commercially available starting materials, as well as the purchase of commercially available chelators related to the above groups (FIG. 1), was initiated. Emphasis was mainly given to heteroaromatic derivatives and, in particular, pyridines because of their acid stability and absorption through the oral route. Thus, L1 was prepared from benzyl maltol using a method similar to that described for mimosine homologues,[8,17] 1-methyl-3-hydroxypyrid-2-one (L2) from a known method,[8,18] 4-methoxy-2-hydroxypyridine-1-oxide (L6) and a novel chelator, 2-hydroxy-4-oxy (2′-methoxyethyl)pyridine-1-oxide (L7), from a known method,[8,19] and the novel 1,4-dihydroxypyrid-2-one (L3: FIG. 1) using a novel method.[8] The above chelators and other commercially available related chelators, including the α-ketohydroxy chelators kojic acid, lawsone, purpurogallin and others,[8] were screened using the methods shown in TABLE 2. Of all the chelators tested in this structure-activity analysis, only L1, L2 and L3 showed promise for further development. L1 was in particular much more effective than the other two. It was anticipated that similar derivatives (FIG. 1) with appropriately selected substituents would also be promising.

Following the original invention and suggestions for designing analogous chelators,[8] over 80 other chelators have been synthesized and tested.[20-22] Of these, the 1-substituted 2-methyl- (or 2-ethyl-) 3-hydroxypyrid-4-ones appeared to be the most promising *in vivo*[23] (TABLE 3). Furthermore, a novel, simple and cheap method of synthesis of L1 and other related 1-substituted 2-methyl- (or 2-ethyl-) 3-hydroxypyrid-4-ones has been developed[22,24,25] which will increase the prospects of making L1 and related drugs available to many countries where chelation treatment is not available because of the high cost of DF.

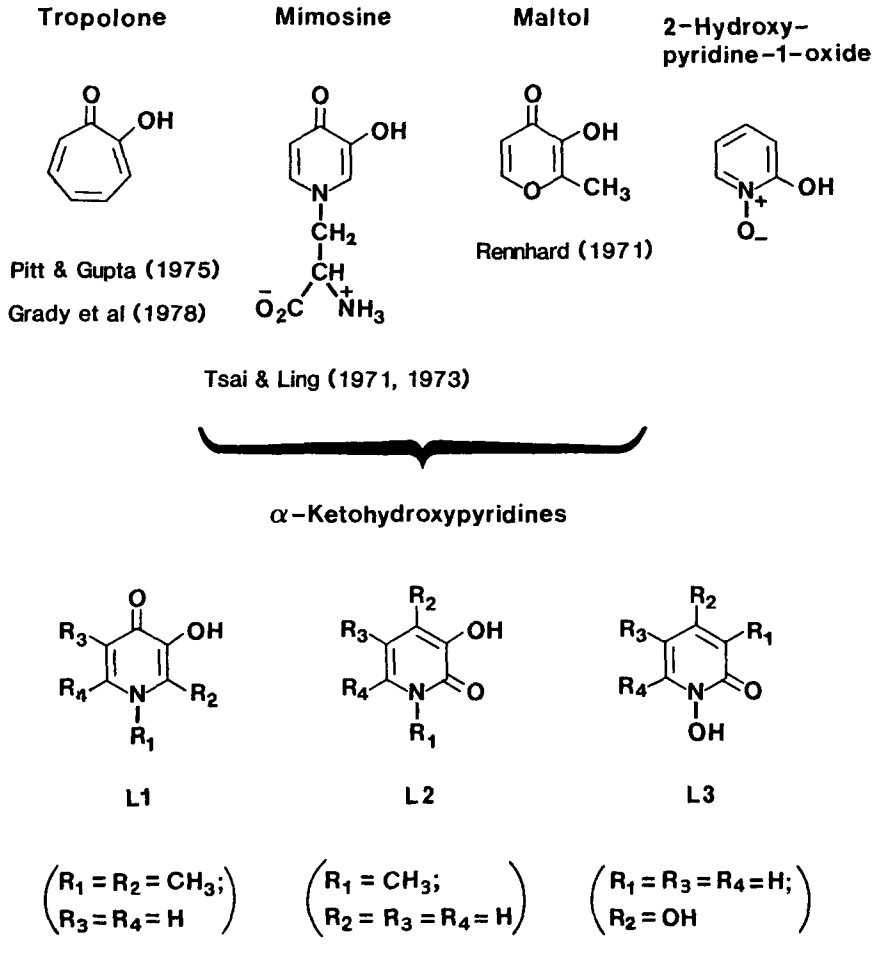


FIGURE 1. The design of L1 and other α-ketohydroxypyridines. Their design originated from mimicking the iron-binding site of the naturally occurring, orally absorbed α-ketohydroxy heteroaromatic chelators tropolone, mimosine, and maltol. (From Kontoghiorghes.[8])

TABLE 2. Design and Development of α-Ketohydroxypyridines

Examination of properties of commercially available α-ketohydroxy and related chelators
Synthesis (known and novel synthetic routes using commercially available reactants)
Structure-activity correlation:
Substituents: electron releasing, neutral, hydrophilic
Solubility, stability, p*K*, lipid/water partition of chelator and iron complexes
Red blood cell uptake of chelator and iron complexes
Iron removal from transferrin/ferritin at physiological pH
Iron removal from mice
Further development of effective chelators: acute, subacute, chronic toxicity in two animal species (one non-rodent), normal and iron-loaded animals
Short-term clinical trials to determine acceptability and efficacy using stepwise dosing (1 week)
Phase II and III clinical trials

DEVELOPMENT OF L1 AND OTHER α-KETOHYDROXYPYRIDINES FOR CLINICAL USE

Once the oral activity of L1 and related chelators had been established,[26–28] the possibility for short-term trials was investigated following subacute animal toxicology in mice, rats, and rabbits. Very few of these chelators, however, fulfilled the major criteria for possible long-term use in humans. Such chelators would need to have a parenteral LD_{50} at least three times higher in comparison to the effective dose anticipated in humans and no major toxicity or mortality long-term. The median lethal dose, the iron removal effect, and the therapeutic efficiency of such chelators are shown in TABLE 3. Of these, L1 and L1NAll[29] appear to have the highest potential for development. In further studies, the long-term effect of some of these chelators administered intragastrically at 200 mg/kg, 5 days a week, in rats was also investigated and compared to intraperitoneal DF. As it can be seen from FIGURE 2

TABLE 3. Therapeutic Efficiency of Orally Effective 1-Substituted 2-Alkyl-3-hydroxypyrid-4-one Iron Chelators

Compound	MLD[a]	% Fe[b]	TE[c]
2-Methyl-3-hydroxypyrid-4-one			
1-methyl- (L1)	650	475	30.87
1-ethyl- (L1NEt)	450	490	22.05
1-(2′-methoxyethyl)- (L1NMeOEt; C52)	350	442	15.47
1-(3′-ethoxypropyl)- (L1NEtOPr)	650	312	20.28
1-allyl- (L1NAll)	350	634	22.22
2-Ethyl-3-hydroxypyrid-4-one			
1-methyl- (EL1)	475	437	20.61
1-ethyl- (EL1NEt; C94)	350	404	14.14
1-(2′-methoxyethyl)- (EL1NMeOEt)	650	303	21.4
1-(3′-ethoxypropyl)- (EL1NEtOPr)	650	320	20.23
1-allyl- (EL1NAll)	450	339	15.25

[a]MLD, median lethal dose in male rats treated intraperitoneally with chelators.

[b]% Fe, percentage iron excretion in iron-loaded, ^{59}Fe-labeled male mice by comparison to controls receiving no chelation. Chelators were administered intragastrically at 250 mg/kg and their effectiveness assessed using a previously described method.[8,20]

[c]TE, therapeutic efficiency (MLD × % Fe × 10^{-4}).

only L1 appears to be safe under these conditions, whereas other chelators such as EL1NEt (C94) and L1NMeOEt (C52), which were also tested by another group and suggested as alternative chelators to L1,[30] caused death for most rats within 2 months and for all rats within 5 months. These two chelators should be excluded from further development, not only because they are lethal long-term, but because they are not as effective as L1 in iron removal. Intraperitoneal DF at the same dose appeared to be safe in the long-term studies, although 2 out of 12 animals died (FIG. 2). The reason for the lower toxicity of L1 and DF in contrast to the other two, lipophilic chelators may be related to the much higher hydrophilicity of L1, DF and their iron complexes, which results in lower partition and accumulation into the tissues and also their higher rate of excretion. Long-term toxicity studies with other promising members of the families of chelators listed in TABLE 3 continues. Moderate decreases in the white blood cell counts of the rats treated with L1 in the long-term studies (8 months) with subacute doses (FIG. 2) were noted, but these were not highly significant when compared to the white cell counts quoted by the animal suppliers (Bantin and

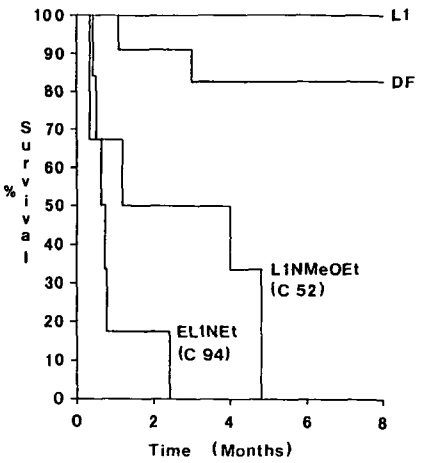


FIGURE 2. The percentage survival of normal rats treated with orally active chelators and DF. DF was administered intraperitoneally to 6 males and 6 females, L1 intragastrically (i.g.) to 6 males and 6 females, EL1NEt (C94) i.g. to 6 males, and L1NMeOEt (C52) i.g. to 6 females. All the rats received a single 200 mg/kg dose five days a week.

Kingman Ltd., Hull, U.K.). Previous reports suggested that these effects were only observed with animals treated with L1,[31,32] but these have now also been observed with other chelators, including EL1NEt and DF.[33] Although there have been only two reported cases (one fatal) of acute myelopathy after high doses of intravenous DF,[34] and also of a neutropenia during an L1 trial,[35] the prospect of these chelators affecting white cell precursors needs further investigation. Previous studies in culture and in animals have shown that iron-containing proteins play a role in the regulation of white cell progenitors, and chelators may therefore affect the development of such cells.[36] Histopathological examination of normal and iron-loaded rats treated with L1 at 200 mg/kg for 6 months revealed no organ damage (liver, spleen, heart, kidneys, lung, pancreas, bone marrow, brain, intestine, stomach, salivary glands, adrenals, eyes, testes, and ovaries) except from mild suppression of the bone marrow (TABLE 4). In the iron-loaded rats only the liver appeared to be depleted of iron.[37] No hypersalivation or other major toxic side effects or mortalities were caused by L1 in

other animals such as mice, rabbits and monkeys at subacute doses (200 mg/kg). For more details on the *in vitro* and *in vivo* properties of L1 see TABLE 4.

PHARMACOLOGY AND TOXICOLOGY OF L1 IN HUMANS

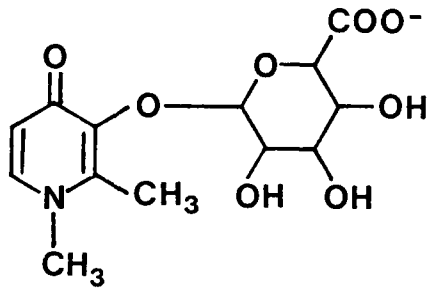
The pharmacological[37a] and other properties of L1 in humans are summarized in TABLE 5. Pharmacological studies in which L1 was monitored in urine, feces and serum using an HPLC technique[38] revealed that L1 is absorbed from the stomach and appears in the serum within minutes of ingestion. The maximum serum concentration of L1 following ingestion of a single 3-g dose is reached within 0.2–2 h, and 85–90% of the drug is eliminated from the serum within 5–6 h of administration. L1 is almost completely metabolized to its glucuronide (FIG. 3) when it is not bound to iron, e.g., in normal individuals. Maximum glucuronide concentration is reached within 2–3 h of ingestion, and the metabolite is progressively eliminated after this period. The iron complex of L1 appears to be eliminated from the serum more slowly than is L1 but faster than is its glucuronide. In pharmacokinetic studies involving 8

TABLE 4. Properties of L1 *In Vitro* and *In Vivo*

Name
1,2-Dimethyl-3-hydroxypyrid-4-one [3-hydroxy-1,2-dimethyl-4(1*H*)pyridinone]
Physicochemical Properties
White crystalline solid, soluble in water (16–18 mg/ml at 24°C). Neutral molecule forming a neutral red 3:1 chelator-iron complex at pH 7.4.
Stability
Shelf-life greater than 3 years. Stable in acidic (pH < 1) and basic (pH > 12) solutions. Iron complex stable in solution for over 2 years.
Interaction with Iron-containing Proteins
Removes iron from ferritin, hemosiderin, and diferric transferrin and lactoferrin. Inhibits cyclooxygenase, lipoxygenase, ribonucleotide reductase and their enzymatic pathways.
Free Radicals
Inhibits free radical formation catalyzed by iron (ferrous or ferric), cyclooxygenase, and lipoxygenase. Prevents post-ischemic cardiac injury (rat hearts).
Interaction with Cells
Inhibits iron uptake by cells such as red cell and lymphocytes. Removes iron from hepatocytes and macrophages. Inhibits DNA synthesis in lymphocytes *in vitro* and in neuroblastoma cell lines.
Effects on Metal Ions in Animals
Increases the excretion of iron from iron-loaded mice, rats, rabbits and monkeys, mainly through the bile. Does not increase iron absorption or the excretion of other metal ions, e.g., Ca, Zn, Mg, and Cu. Increases the excretion of Al in Al-loaded animals.
Toxicity
Acute: median lethal dose range, 600–700 mg/kg i.p.; 2–3 g/kg i.g. Subacute: 200 mg/kg/day, 60–240 days, causes moderate decrease of the white cell count in rats and mice and mild hypocellularity of the bone marrow, affecting mainly white cell progenitors. Chronic: 60 mg/kg, 200 days, produces no decrease in the white cell count. Causes hypersalivation in rats. Reports of causing hyperplasia of the adrenals, increases in cholesterol levels, and interaction with barbiturates are not confirmed. L1 and its iron complex are not mutagenic.

TABLE 5. Properties of L1 in Humans

Clinical Trials
130 persons received L1 for a maximum period of 15 months, a maximum single dose of 4 g/24 h, maximum divided doses of 16 g/24 h, resulting in a maximum level of urinary iron excretion of 325 mg/24 h.
Pharmacology
Patients differ in their clearance and metabolism of L1. Absorption is from the stomach (half-life 1–5 min). It appears in blood within 5–10 min (half-life of serum elimination 47–134 min). There is maximum serum elimination of 85–90% within 5–6 h. Maximum serum levels are 87–450 μ*M* per 3-g dose (35–75 mg/kg). It enters into cells of the liver and other organs. It is metabolized to a glucuronide, which appears in blood within 15–20 min and is eliminated as for L1. Urinary excretion of L1, L1-iron complex, and L1 glucuronide accounts for nearly 100% recovery of the administered dose; it is absent from fecal excretions. Iron is removed mainly from serum, serum transferrin, and the liver.
Toxicity
There are no established toxic side effects. Adverse observations include (*a*) transient Lw antibody and agranulocytosis in a Blackfan-Diamond patient, (*b*) transient joint and musculoskeletal pains in 10 thalassemia and 1 myelodysplasia patient, (*c*) increased titer of the rheumatoid factor in 2 patients, (*d*) increased blood requirements of 1 non-splenectomized thalassemia patient, and (*e*) temporary gastric intolerance in 5 patients.
Other Effects
There was improvement of the arthritic condition of 1 myelodysplasia patient, increased production of hemoglobin in rheumatoid arthritis patients with the anemia of chronic disease, and increased excretion of aluminum in renal dialysis patients.



Glucuronide of L1

FIGURE 3. Structure of L1 glucuronide.

patients,[37a] L1 was excreted in the urine mainly in its metabolized form, with the remainder as the iron complex. Treatment of urine or serum samples with β-glucuronidase[15] resulted in the conversion of the L1 glucuronide to L1 and accounted for almost 93% recovery of the administered dose in the urine of 6 patients. One myelodysplasia patient did not appear to have the L1 glucuronide in her urine. Despite this, iron excretion was related to the level of her iron stores and not to the level of free, non-metabolized L1. Absence of L1 in feces confirmed that L1 and its iron complex are solely excreted through the urine. This observation coincides with the absence of increased fecal iron excretion in the iron-balance

studies of patients who received up to a maximum of 9 g (3 × 3-g doses) per day of L1.[39,40,40a] It could be envisaged from the pharmacokinetic studies that the iron removal efficiency of L1 will increase by repeated administrations of 2–3-g doses every 4–6 h. The observed variations in the clearance and metabolism of L1 indicate the need for the determination of the pharmacokinetic profile of patients taking part in intensive chelation studies in order to avoid possible toxicity due to accumulation of the drug or its metabolite(s).

The only possible side effects which have emerged in the multicenter studies and which appear to affect a few patients are transient joint and musculoskeletal pains (TABLE 5). These symptoms have been previously reported in the United Kingdom[5,40b] and appear to have occurred in some patients in India. Their cause is not known, but it may be related to the trapping and accumulation of the iron complex or the metabolite(s) of L1 in the synovial fluid or muscle tissues, thus affecting membrane fluidity and fluid viscosity, resulting in inflammation and tissue damage. Environmental factors such as interaction with other metals in food or in biological fluids, the racial origin of patients, and drug impurities may also be relevant. Other adverse effects reported, such as agranulocytosis in one Blackfan-Diamond patient,[35] and those observed in animals treated with subacute doses of L1 should also be monitored. Overall, however, the absence of major toxicity in patients who were treated with L1 for up to 15 months, with single doses of up to 4 g/day and divided doses of up to 16 g/day, is remarkable. Very few drugs are known to be administered at such doses and to have no major toxic side effects.

RESULTS OF IRON CHELATION BY L1 IN HUMANS

One of the major criteria for any iron chelator intended to replace DF is its ability to decrease the iron load of chronically transfused patients and to maintain it at low, safe levels, close to those of normal individuals. In iron-excretion balance studies involving 6 thalassaemia patients, L1 did not increase fecal iron excretion, even at doses of 9 g/day (divided into 3 × 3 g).[40,40a] Furthermore, studies in humans and in animals[40,40c] have shown that L1 does not promote the absorption of iron. The measurements of iron and L1 in the urine appear to be good indicators of the overall elimination of iron. This net iron loss can be compared to the net intake of iron from transfusions, which is estimated to be approximately 25 mg iron per day in adult thalassaemia patients.

Iron excretion caused by L1 is related to three main factors: the dose, the frequency of administration, and the iron load of the patient. In general, the higher these factors are, the more iron will be excreted per given dose of L1. This relationship is depicted in FIGURE 4, where urinary iron excretion in response to effective single or repeated doses of L1 (25–160 mg/kg/day) seems to depend on the iron load of the patients, expressed as units of red blood cells (RBC) transfused. A dose of 2 × 2 g, for example, in 10 rheumatoid arthritis patients who did not receive any transfusions, resulted in less than 1 mg of urinary iron.[41] In contrast, in an iron-loaded thalassemia patient (>700 RBC units transfused) the same dose resulted in 66 mg of urinary iron. Repeated administration every 3–4 h of L1 causes iron excretion which is always higher than that with a single dose in the same patient. Urinary iron exceeded 25 mg (30–126 mg) with a 2 × 3-g dose of L1 in almost all 13 transfusional iron–loaded patients who were treated in the United Kingdom for over a month.[40a] The exception was one red cell aplasia patient, who in total received 47 units of RBC and excreted only 18.2 mg of urinary iron. In comparative studies between L1 and DF in transfusional iron–loaded patients, urinary iron was equiva-

lent with both drugs for 2–3-g single daily doses[3,4] or, in another study, 50–75 mg/kg given in divided doses.[42] In iron-excretion balance studies, DF increased fecal iron excretion at 3-g or 2 × 2-g daily doses but not at 2-g or 2.5-g doses. The net amount of fecal iron excretion was equivalent to 10–25% of the net increase in urinary iron excretion.[40a] The repeated administration of 3 × 3 g of L1 per day resulted in most cases in a urinary iron excretion 2–2.5 times higher (65–257 mg iron/day) than the total iron excretion caused by DF. In considering the long-term applications of L1 and DF it appears that the former will be more efficacious because of the ease of repeated administrations in a single day and also during the weekends.

In the long-term studies in the United Kingdom, L1 was administered for a total of 15 months to two patients, 13 months to one patient, 8 months to one patient, 4–5.5 months to four patients, and 1–2 months to five patients. Administration of

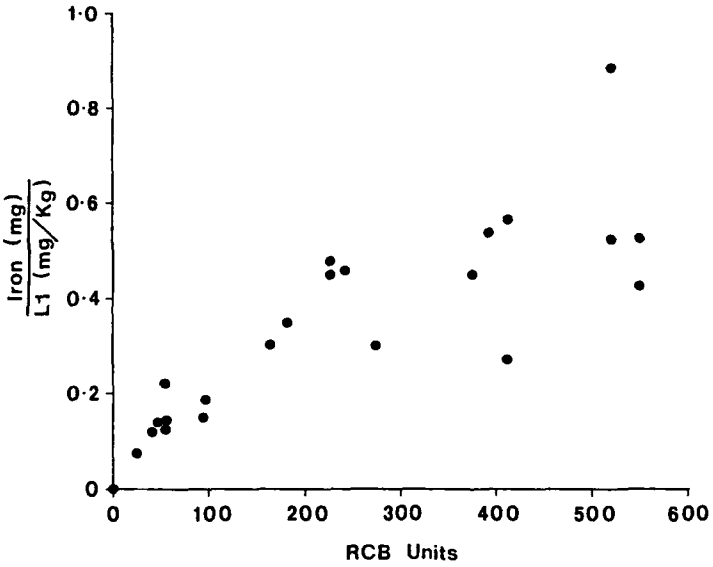


FIGURE 4. The effect of iron excretion caused by L1 in patients with variable iron stores. Each point represents a patient who received L1 at single or repeated doses of 25–160 mg/kg. RBC Units, red blood cell units transfused.

single 3-g or of 2 × 2-g doses of L1 in the first 5–6 months resulted in three of four patients excreting less, and one more, than 25 mg iron/day. After this period, when 2 × 3-g or higher doses were used, iron excretion far exceeded 25 mg. Overall, there was no change in the serum ferritin of these patients. However, a large drop in serum ferritin was observed in over 80% of the patients who took L1 in India for three months but had not received previous DF treatment (M. B. Agarwal, personal communication) and also in four out of eight patients in Switzerland (P. Tondury, personal communication) who have been taking L1 for 10 months and have had previous DF treatment. Reduction in serum ferritin was also observed in the rheumatoid arthritis patients who took 2 × 2 g L1 for three weeks.[41] Although L1 and DF may have different effects on serum ferritin synthesis, it would be surprising if

long-term treatment with effective doses of L1 did not result in a decrease in serum ferritin in all the patients.

In conclusion, L1 is an orally active chelator which can be used effectively for the treatment of transfusional iron overload in thalassemia. Its low cost and, as yet, lack of apparent serious toxicity increases the prospects for its use in the treatment of transfusional iron-loaded patients worldwide. Further long-term studies both in animals and patients are required to determine possible side effects and the long-term efficacy of L1.

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New Orally Effective Iron Chelators

Animal Studies[a]

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Despite its proven efficacy, deferoxamine (DF) suffers from a number of serious limitations, such as short duration of action, poor intestinal absorption, and high price. New, inexpensive, and orally effective iron chelators would not only make long-term chelating therapy more acceptable, but would also increase its efficacy by generating a continuous supply of circulating drug by virtue of its slow but uninterrupted intestinal absorption.

AVAILABLE MODELS

Both *in vitro* and *in vivo* models have been employed for evaluating new chelating agents. Hepatocyte and myocardial cell cultures have been used extensively for *in vitro* studies of iron chelators.[1-5] These systems permit assessment of the ability of the chelators to interfere with cellular iron uptake and subsequent incorporation into ferritin and other intracellular compartments. More importantly, the mobilization of

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cellular iron following *in vitro* treatment with iron-chelating drugs can be monitored directly. Limited information on cytotoxicity may also be derived from cell morphology, interference with protein synthesis, or release of cytoplasmic enzymes into the extracellular medium. The advantage of the *in vitro* screening system is its simplicity, allowing the screening of large numbers of drugs in a relatively short time with complete control of the extracellular environment. There are, however, some important limitations. Hepatocytes and heart cells are not an exclusive source of chelatable iron, and other important sources of iron such as the reticuloendothelial (RE) system would be totally missed in a liver or heart cell culture model.[6] Another serious limitation is the inability to identify drugs which are converted into the active metabolite *in vivo* before reaching their target organ. Finally, intestinal absorption, one of the most important features of the new generation of iron chelators, obviously cannot be studied *in vitro*. In spite of these limitations, studies performed in cell cultures have yielded useful information on the chelating efficiency of a great number of compounds. Such data must be supplemented by *in vivo* studies.

Because of their low price and ease of handling, mice and rats have been used extensively for the *in vivo* study of iron chelators. As only a minor fraction of the total body iron is normally available for chelation, various methods have been used in order to increase the effectiveness of this model system and to simulate the clinical condition of transfusional iron overload. Hypertransfusion was used by several investigators, employing various methods.[7-9] Others have used simple colloidal iron injections.[10] The final common effect of these procedures is to increase iron stores and thus to increase the response to chelating therapy. An alternative method to increase the sensitivity and specificity of the *in vivo* models is the injection of radioiron-labeled compounds. Some of these, such as ^{59}Fe-ferritin or ^{59}Fe-labeled heat-damaged erythrocytes (^{59}Fe-DRBC) can be used as selective radioiron probes to label hepatocellular or RE iron stores, respectively.[9] The use of such selective radioiron probes obviates the need for hypertransfusion and permits the evaluation of a large number of iron-chelating agents with considerable savings in time and expense.[11]

In vivo models are capable of providing important data on such aspects as oral versus parenteral efficacy, net balance between iron absorption and excretion as reflected in the magnitude of residual iron stores in a variety of organs, promotion of radioiron excretion from various selectively labeled storage iron pools, and a large number of clinical, morphologic and biochemical parameters for recognizing drug toxicity. Important information may also be obtained on the route of iron excretion which, as a rule, is preferential renal clearance of the more hydrophilic compounds and biliary excretion of the hydrophobic, lipid-soluble molecules.

Over the last decade, several hundred candidate compounds have been screened using *in vitro* and *in vivo* models or a combination of both. These studies have led to the identification of several interesting compounds of possible clinical usefulness. For the sake of convenience and simplicity, the following discussion will be limited to the most outstanding of these compounds studied in the authors' laboratories, all of which are effective by oral administration. These compounds are the polyanionic amines, the aryl hydrazones, and the hydroxypyridin-4-ones.

POLYANIONIC AMINES

A systematic search for useful new iron-chelating compounds (TABLE 1) has led to the rediscovery of two very powerful polyanionic amines synthesized over 20 years ago by the group led by Martell:[7,8,12,13] *N,N'*-ethylenebis(2-hydroxyphenylglycine)

TABLE 1. Excretion and Organ Distribution of Radioiron-Drug Complex in Rats

Drug	Distribution (% ± SD)				
	Urine	Stool	Blood	Liver	Residual
Control	0.1 ± 0	4.8 ± 0.1	27.1 ± 3.6	12.4 ± 1.7	54.6 ± 2.6
Salicylhydroxamic acid	0.3 ± 0	3.5 ± 0.2	31.8 ± 0.5	11.5 ± 1.2	52.9 ± 3.8
Salicylaldomine	13.1 ± 0.3	8.1 ± 0.2	15.4 ± 0.3	7.8 ± 0.3	55.6 ± 1.2
8-Hydroxyquinoline	0.1 ± 0	3.6 ± 0.2	24.6 ± 3.2	16.7 ± 3.2	55.0 ± 5.7
Pyrocatechol violet	0.4 ± 0	3.7 ± 0	22.6 ± 0.5	11.6 ± 0.5	61.7 ± 0.7
4,5-Benzotropolone	1.9 ± 0.7	4.9 ± 1.9	37.3 ± 2.3	13.7 ± 0.7	41.6 ± 1.9
2,3-Dihydroxynaphthalene	1.6 ± 0.1	6.0 ± 0.1	25.3 ± 0.9	9.1 ± 1.5	58.0 ± 1.0
2-Piperidinomethyl-3-hydroxy-6-hydroxymethyl-4*H*-pyran-4-one	4.4 ± 0.1	10.7 ± 0.3	35.1 ± 2.5	11.2 ± 2.1	38.6 ± 1.9
2,3-Dihydroxybenzoic acid	5.1 ± 0.1	9.4 ± 0.2	21.7 ± 0.2	8.9 ± 0.2	54.9 ± 1.3
2,5-Dihydroxybenzoic acid	4.8 ± 0.2	17.8 ± 0.6	15.6 ± 1.1	9.7 ± 1.0	52.1 ± 1.8
Cholyhydroxamic acid	8.1 ± 0.2	11.2 ± 0.3	27.9 ± 0.1	8.3 ± 0.7	44.5 ± 1.2
m-Chlorobenzhydroxamic acid	0	4.4 ± 0.1	17.2 ± 1.0	11.6 ± 0.4	66.8 ± 1.0
N-Methyl-*m*-chlorobenzhydroxamic acid	0.4 ± 0	15.1 ± 0.2	18.6 ± 0.4	8.8 ± 1.2	57.1 ± 0.7
N^2,N^9-bis(6-kojylmethyl)-2,9-diazodecane	12.4 ± 0.1	10.5 ± 0.1	39.2 ± 3.7	12.8 ± 0.1	25.1 ± 0.3
N^2,N^5-bis(6-kojylmethyl)-2,5-diazohexane	8.5 ± 0.4	12.7 ± 0.6	43.5 ± 1.7	13.0 ± 0.7	22.3 ± 1.0
N,N'-bis(6-kojylmethyl)-piperazine	9.9 ± 0.2	11.7 ± 0.2	27.0 ± 2.3	10.4 ± 1.4	41.0 ± 0.8
Tetrasodium-*m*-tetra(4-sulfonatophenyl)-porphine	0	6.9 ± 0.2	11.0 ± 0.6	9.2 ± 0.8	72.9 ± 2.3
Tropolone	0	29.4 ± 4.1	12.1 ± 2.5	10.2 ± 5.4	48.3 ± 6.8
Pyridoxyl isonicotinoyl hydrazone (PIH)	4.3 ± 0.4	32.8 ± 5.9	32.0 ± 3.1	9.6 ± 1.3	20.3 ± 11.2
Imidazole acetic acid	24.7 ± 0.5	11.3 ± 0.2	2.9 ± 0.4	7.4 ± 0.5	53.7 ± 1.1
N',N^3-bis(2,3-dihydroxybenzoyl)spermidine HCl	7.2 ± 0.1	25.2 ± 0.5	1.7 ± 0.1	2.4 ± 0.1	63.5 ± 1.4
N,N'-bis(2,3-dihydroxybenzoyl)-1,6-diaminohexane	17.3 ± 1.8	47.3 ± 4.9	8.0 ± 1.2	6.5 ± 0.1	20.9 ± 6.1
N,N'-bis(2-picomeconylmethyl) piperazine	23.0 ± 0.8	23.7 ± 0.9	18.1 ± 1.8	11.9 ± 0.4	23.3 ± 0.8
N',N^5,N^{10}-tris(2,3-dihydroxy-5-sulfobenzoyl)triazodecane	28.8 ± 0.1	24.6 ± 0.1	11.0 ± 0.1	13.0 ± 0.2	22.6 ± 0.2
1,3,5-tris(N,N',N^{11}-2,3-dihydroxybenzoyl)-aminomethyl-benzene	17.2 ± 0.5	49.8 ± 1.4	3.0 ± 0.1	6.6 ± 1.6	23.4 ± 0.6
Ethylenediamine-*N,N'*-bis(*o*-hydroxyphenylglycine) (EHPG)	41.7 ± 0.8	22.2 ± 1.4	15.3 ± 2.0	7.0 ± 1.1	13.8 ± 1.6
Ethylenediamine-*N,N'*-bis(*o*-hydroxyphenylglycine) dimethyl ester	35.9 ± 2.2	28.9 ± 1.7	12.9 ± 1.3	5.2 ± 0.6	17.1 ± 2.5
N,N'-bis(2-hydroxybenzyl)ethylenediamine diacetic acid (HBED)	24.8 ± 1.7	69.4 ± 4.8	2.5 ± 0.3	0.8 ± 0.1	2.5 ± 0.5
N,N'-bis(2-hydroxybenzyl)ethylenediamine diacetic acid-dimethyl ester	22.8 ± 0.5	62.0 ± 1.3	7.2 ± 0.7	1.9 ± 0.2	6.1 ± 0.4
Ethylenediamine-*N,N'*-bis(2-hydroxy-5-carboxyphenylglycine)	6.0 ± 1.0	60.1 ± 10.3	8.3 ± 1.3	3.3 ± 0.3	22.3 ± 4.6
2-Pyridine carboxaldehyde 2-pyridyl hydrazone	6.8 ± 0.2	47.1 ± 1.4	5.1 ± 0.4	4.9 ± 0.1	36.1 ± 1.1
Picolinaldehyde thiosemicarbazone	56.1 ± 6.1	29.9 ± 2.1	2.5 ± 0.4	7.9 ± 2.9	3.6 ± 1.7
Hydroxamic acid polymer	38.7 ± 0.4	4.3 ± 0.1	11.0 ± 0.2	8.7 ± 0.5	37.3 ± 0.3
N-Acetyldesferrioxamine	53.1 ± 2.0	16.2 ± 0.6	12.8 ± 1.4	4.5 ± 0.2	13.4 ± 0.9
N-3-Pyridylaminocarbonyl desferrioxamine	30.1 ± 1.0	40.0 ± 1.3	11.3 ± 1.5	4.0 ± 0.2	14.6 ± 0.5
Desferrioxamine E	62.5 ± 6.4	24.5 ± 2.4	4.1 ± 0	1.7 ± 0.1	7.2 ± 0.7
Desferrioxamine B	69.5 ± 7.6	5.5 ± 0.4	7.9 ± 1.2	3.3 ± 1.3	13.8 ± 5.9

(EHPG) and *N,N'*-bis(2-hydroxybenzoyl) ethylenediamine-*N,N'*-diacetic acid (HBED). They both form hexadentate ligands with ferric iron by their secondary or tertiary nitrogens and their hydroxyl and carboxyl groups. The affinity constant for iron of EHPG is 33.9 and that of HBED is 39.6.[14,15] Their affinity for other metals is relatively low. Conversion of the carboxylic groups of HBED to methyl esters[16] results in a marked improvement of its intestinal absorption and a further increase in iron excretion.

Our studies in hypertransfused rats injected with ^{59}Fe-ferritin to label hepatocellular iron stores have shown an accurate inverse relation between biliary iron excretion and residual hepatic radioactivity (FIG. 1). Fecal radioiron excretion (% of injected dose) following a single intramuscular (i.m.) injection of dimethyl-HBED, 200 mg/kg, was 80%, with a simultaneous reduction in hepatic radioactivity from 88% in controls to 8%. Dose-response relations shown in FIGURE 2 indicate that at the dose range of 25 to 50 mg/kg, HBED and dimethyl HBED were 12 to 15 times more effective than DF. Finally, chemical measurements have shown that a single dose of dimethyl-HBED, 200 mg/kg i.m., reduced hepatic non-heme iron stores from 2247 ± 185 μg/liver in controls to 632 ± 59, and ferritin iron stores from 1082 ± 62 to 280 ± 90 μg.[9,17] Animal studies involving prolonged EHPG administration disclosed significant toxicity manifested in weight loss, anemia, and hepatic and pulmonary damage.[18,19] In contrast, HBED and dimethyl-HBED are remarkably non-toxic, and their LD_{50} is in excess of 800 mg/kg as against 100 for EHPG.[7] Clearly, dimethyl-HBED is one of the most promising oral iron chelators identified so far; and if its low toxicity in rodents is confirmed by long-term toxicity studies in higher animal species, it may represent a significant advance in the development of drugs for the management of clinical iron overload.

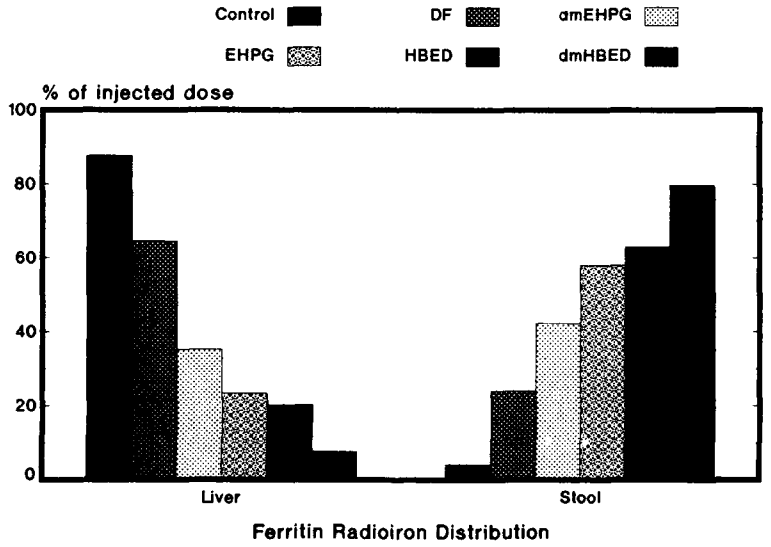

FIGURE 1. Phenolic ethylenediamine derivatives. Residual hepatic radioactivity (*left*) and cumulative fecal radioiron excretion (*right*) in hypertransfused rats labeled with ^{59}Fe-ferritin intravenously and treated by a single intramuscular injection of 200 mg/kg DF, EHPG, HBED, or their dimethyl (dm) derivatives. Results expressed as % of total injected radioactivity. Measurements performed on day 6.

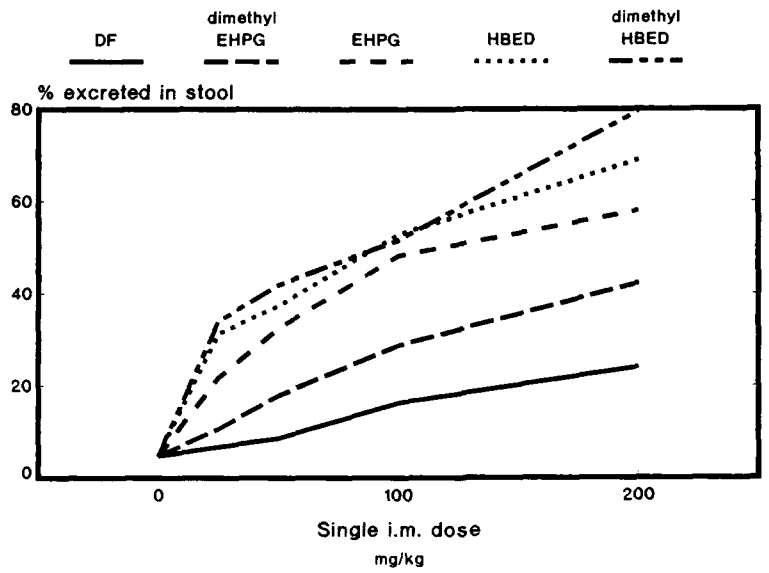

FIGURE 2. Relation of dose to radioiron excretion. Study of dose-response relation in hypertransfused rats labeled with ^{59}Fe-ferritin intravenously and treated by a single intramuscular (i.m.) injection of 25, 50, 100, or 200 mg/kg drug. Cumulative fecal excretion expressed as % of total injected radioactivity. Measurements performed on day 6.

THE ARYL HYDRAZONES

The prototype of the aryl hydrazone family of compounds is pyridoxal isonicotinoyl hydrazone (PIH), introduced by Ponka *et al.*, who recognized its ability to mobilize iron from ^{59}Fe-labeled reticulocytes.[20,21] In hypertransfused rats,[22-24] PIH is able to mobilize storage iron, in addition to reticulocyte iron. Its oral and parenteral effectiveness are similar. PIH is able to remove parenchymal and RE iron with equal efficiency, and practically all the chelated iron is excreted via the bile.[22] Its *in vivo* chelating efficiency is equal to, or slightly better than, that of DF. No evidence of toxicity has been found in any of the hepatocyte culture or the animal studies involving PIH[5,7,22] at doses up to 500 mg/kg/day. However, results of long-term oral treatment with PIH in rats have been disappointing:[25] At the end of 10 weeks of treatment, no reduction in hepatic iron stores or in whole-body radioactivity beyond that found in control animals could be demonstrated. This has been attributed in part to interaction with intraluminal food iron prior to drug absorption. It should be emphasized, however, that the iron content and the magnitude of food iron absorption in rodents is greatly in excess of that in man.[26] Ultimate judgement on the therapeutic effectiveness of oral PIH should be withheld until such studies are repeated in human subjects.

Other Schiff base compounds are readily formed by pyridoxal, and a large number of such derivatives has been studied.[21,22,24,25] In a collaborative effort, we have studied over 40 such new derivatives in recent years.[22,27,28] Most of these were inferior to PIH both in their ability to promote iron excretion and in their oral effectiveness. However, more recently some promising new derivatives have been identified.

FIGURE 3 illustrates the effect of some of these new pyridoxal pyrimidinyl hydrazones (PPH) on fecal iron excretion and residual hepatocellular radioactivity. The most effective of these, pyridoxal-pyrimidinyl-ethoxycarbonyl methbromide (PPH15), is able to remove 79% of hepatocellular radioiron stores following a single parenteral dose of 200 mg/kg, and its oral effectiveness exceeds that of parenteral DF. Results of preliminary toxicity studies in rats are encouraging. However, in view of the disappointment experienced in the past with a number of promising iron-chelating compounds, any statements concerning the suitability of the new PIH derivatives for clinical use prior to the completion of detailed toxicity trials would be inappropriate.

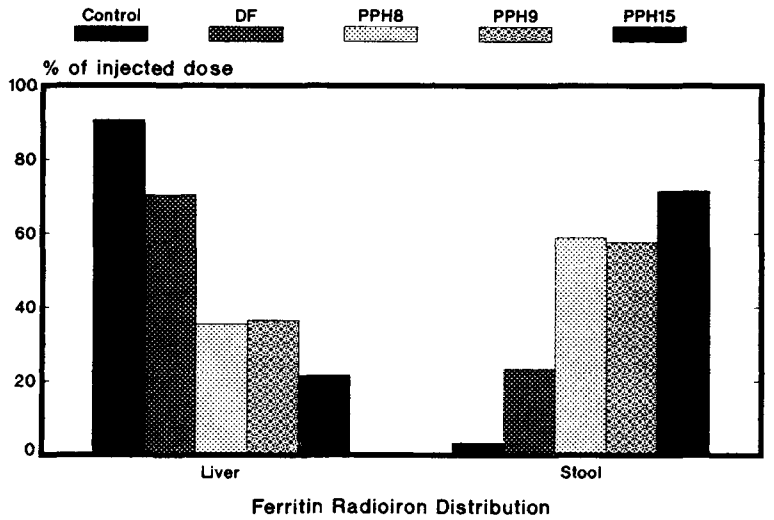

FIGURE 3. New pyridoxal derivatives. Residual hepatic radioactivity (*left*) and cumulative fecal radioiron excretion (*right*) in hypertransfused rats labeled with ^{59}Fe-ferritin intravenously and treated by a single intramuscular injection of 200 mg/kg DF, PPH8, PPH9, or PPH15. Results expressed as % of total injected radioactivity. Measurements performed on day 6. PPH, pyridoxal pyrimidinyl hydrazone.

THE HYDROXYPYRIDIN-4-ONES

The hydroxypyridin-4-ones, a new class of iron chelators, have been designed by Hider and Kontoghiorghes to permit optimal intestinal absorption.[4,10,29-35] The 3-hydroxypyridin-4-ones have emerged as the best candidate drugs in this class. These bidentate chelators bind to iron in a 3:1 ratio with a stability constant of 37, about six orders of magnitude higher than that of DF. Increasing the size of the alkyl function substitution on the nitrogen atom of the ring enhances their lipophilicity without altering their affinity for iron.[4,10,34,36] Low lipophilicity is associated with reduced chelating efficiency, whereas high lipophilicity is associated with increased toxicity. The chelating properties of the hydroxypyridin-4-ones have been studied both *in vitro* in hepatocyte cultures and *in vivo* in several species of animals, including mice, rats, and rabbits.

TABLE 2. Effect of Hydroxypyridin-4-one Treatment on Rat Heart Cells in Culture

Treatment[a]	*n*	^{59}Fe Release (% ± SD)	LDH Release (%)[b]
Control	3	0	2.84
DF	3	78.3 ± 1.1	0.47
CP20	3	82.7 ± 0.4	0.18
CP40	3	81.6 ± 0.2	0.97
CP51	3	86.1 ± 0.5	1.44
CP94	3	87.9 ± 1.1	1.12
CP96	3	87.3 ± 0.2	3.80

[a]24-h exposure to 0.3 m*M* concentration of indicated compound.
[b]LDH, lactate dehydrogenase.

In view of the detailed presentations by HIDER and by KONTOGHIORGHES appearing elsewhere in this volume, our comments about these compounds will be limited to the information generated at our own laboratories with hydroxypyridin-4-ones supplied by Dr. Hider. We have taken advantage of our *in vitro* system of cultured heart cells to explore the ability of the various hydroxypyridin-4-ones (CP20 to CP96) to remove iron directly from myocardial cells (TABLE 2). Cells were pretreated by 24-h incubation with radioiron-labeled ferric ammonium citrate at a concentration of 20 μg/ml and then exposed for a further 24 h to 0.3 m*M* solutions of the compounds listed in TABLE 2. All the pyridinones were more effective at removing radioiron from iron-loaded myocardial cells than DF. Lactate dehydrogenase (LDH) release into the culture medium, used as an indicator of cellular damage, was negligible. The most effective chelators in these *in vitro* studies were CP51, CP94, and CP96.

These *in vitro* studies were supplemented by storage iron measurements in rats given DF or a CP compound by subcutaneous injections every 8 h at a dose of 300 mg/kg/day for 13 consecutive days (TABLE 3). These studies showed a clear distinction between CP20, CP38 and CP40, which were unable to reduce liver iron stores, and CP51, CP94 and CP96, which were effective in depleting hepatic iron. However, increased *in vivo* chelating efficiency was associated with increased toxicity, as manifested in weight loss which, in the case of CP51, was quite remarkable. Of the compounds tested, CP94 represented the optimal combination of increased effi-

TABLE 3. Effect of Hydroxypyridin-4-one Therapy on Weight and Iron Status In Rats

Treatment[a]	*n*	Day 0 Body Weight (g, mean ± SD)	Day 13 (mean ± SD) Body Weight (g)	Liver Weight (g)	Non-heme Fe (μg/liver)	Serum Fe (μg/dl)
Control	6	136 ± 7	174 ± 12	7.88 ± 0.75	1323 ± 297	542 ± 79
CP20	6	142 ± 5	178 ± 13	9.15 ± 1.16	1323 ± 274	519 ± 200
CP38	6	137 ± 6	167 ± 9	8.34 ± 0.75	1326 ± 355	404 ± 55
CP40	6	139 ± 2	169 ± 5	7.73 ± 1.18	1495 ± 418	412 ± 181
CP51	6	138 ± 8	110 ± 19	5.73 ± 1.50	463 ± 118	660 ± 76
CP94	6	142 ± 6	146 ± 17	6.05 ± 0.30	578 ± 137	513 ± 179
CP96	6	137 ± 8	148 ± 18	5.82 ± 1.00	539 ± 157	365 ± 128
DF	6	130 ± 4	161 ± 10	6.08 ± 0.59	677 ± 142	333 ± 83

[a]Subcutaneous injections at 8-h intervals, 300 mg/kg/day for 13 consecutive days.

ciency and minimal toxicity. The relative efficiency of CP94 in depleting liver iron stores by oral compared to parenteral administration was 82%.

COMMENTS

None of the compounds discussed is immediately available for replacing DF as an effective oral chelator for clinical use. The major gap between research and clinical application is the need for rigorous, time-consuming, and expensive formal toxicity studies. Such studies are, however, beyond the means of individual scientists and will only be performed when industrial or governmental funding is made available for such purpose. Past experience with a number of promising chelating compounds indicates that unless, and until, these new drugs have emerged successfully from rigorous toxicity testing, it would be unwise to make public announcements regarding their availability for clinical use in the immediate future.[37,38]

A number of considerations should be borne in mind in the selection of an optimal compound for future use in iron chelating therapy. Such a compound should have improved *in vivo* chelating activity, but this is not an absolute need since a new drug with an oral effectiveness comparable to that of parenteral DF would be quite acceptable. High intestinal absorption is mandatory, but care should be taken that *in situ* chelation of luminal iron within the gut does not enhance iron absorption instead of promoting its excretion. A preferential interaction with liver and heart cell iron deposits would be desirable, since iron accumulated within such cells is more harmful than that in RE cells. This in turn is a function of the partition coefficient (lipid versus water solubility), which in some compounds, such as the hydroxypyridin-4-ones, may be regulated at will by altering the side chain of the molecule. An ideal compound should also have a prolonged effect to ensure the continued presence of the chelator in the circulation, which in turn would protect tissues from the accumulation of non-transferrin iron in plasma. This objective could be easier to achieve with an oral medication, possibly in slow-release tablets, than with parenteral drugs which are rapidly cleared from the blood. Finally, an improved iron chelator should have low toxicity and should be inexpensive, in order to make it available for a maximal number of patients.

Although we do not yet have a drug available for large-scale clinical use to replace DF, significant progress has been made in recent years. The hope that some of the compounds discussed above may prove suitable for long-term clinical use is based on encouraging preliminary results. With increasing interest on the part of the pharmaceutical industry on the one hand, and public awareness of the need for developing improved iron chelating medications on the other hand, it is reasonable to expect that such drugs may indeed be introduced for clinical use in the near future.

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HBED: A Potential Oral Iron Chelator[a]

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It has become increasingly clear that excess iron plays a significant role in the pathogenesis of many disease states. Accordingly, there is a genuine need for improved methods of iron-chelation therapy. While desferrioxamine (DFO) is remarkably safe and effective, the fact that it must be administered parenterally for prolonged periods precludes consideration of its widespread use in a number of chronic situations, such as rheumatoid arthritis, where an iron chelator might be helpful.[1,2] The need for an orally effective drug has been recognized for many years, especially for use in treating the secondary iron overload which develops in those with β-thalassemia major.[3,4] Compliance with the daily use of DFO therapy among this patient population is a major problem.[5,6] While a wide variety of both naturally occurring and synthetic iron-chelating agents is available,[7-10] attempts to develop an oral chelator have been frustrated by factors such as poor gastrointestinal absorption, low bioefficiency, and evidence of toxicity due to either the chelator itself or redistribution of iron within the body.[10]

Attention is now focused on four types of compounds. Derivatives of desferrioxamine designed to enhance oral absorption have an obvious appeal. While some progress has been made with such prodrugs, a number of technical problems have hindered their development (H. H. Peter, personal communication). The discovery by Ponka *et al.*[11] that pyridoxal isonicotinoyl hydrazone (PIH) causes the mobilization of iron from reticulocytes prompted evaluation of many related phenolic hydrazones as potential drugs.[12,13] While several compounds have proven to be more effective than PIH in animal models of iron overload, only the latter has been tested clinically. The results of preliminary studies suggest that use of PIH will not bring about negative iron balance in transfusion-dependent patients, although efforts to increase its bioavailability continue (G. Brittenham, personal communication). 1,2-Dimethyl-3-hydroxypyrid-4-one (DMHP) has also emerged as a potential drug candidate.[14,15] However, a subsequent comparison of DFO (50 mg/kg infused over 12 h) and DMHP (50 mg/kg orally divided into three daily doses) in 10 poorly compliant thalassemia patients revealed that DMHP was clearly superior to DFO in only one patient.[16] In four others the two drugs were comparable; DFO caused significantly greater excretion of urinary iron in the remaining patients. While more extensive clinical trials of DMHP are now underway, serious questions remain regarding both its

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efficacy and safety.[17] It is hoped that the studies now in progress will resolve these issues.

A fourth class of compounds, phenolic aminocarboxylates, is also being actively investigated. One compound in particular, *N,N'*-bis(*o*-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid (HBED), continues to show great promise after years of *in vitro* (Ref. 18 and P. Ponka, unpublished observations) and *in vivo*[18-20] testing. Apart from being a hexadentate ligand, HBED does not resemble any of the naturally occurring siderophores. It forms a 1:1 complex with ferric ion, each of the phenolic, amino and carboxylic acid groups being involved in metal binding. Its affinity for iron ($\log \beta = 40$) is approximately nine orders of magnitude greater than that of DFO[7,9,21]; its affinity for other physiologically important metals, such as calcium, magnesium, copper, and zinc, is considerably lower.[21] Development of HBED as a drug was slowed for some time for lack of a cost-effective method of synthesis. This has now been achieved, and kilogram quantities are available at less than half the cost of DFO.

The potential of HBED was first demonstrated using the hypertransfused rat model of iron overload.[18] This model utilizes female Sprague-Dawley rats overloaded with iron via intraperitoneal (i.p.) injections of heat-damaged rat erythrocytes. During drug screening, groups of six iron-overloaded rats are placed in individual metabolic cages and fed a low-iron (< 10 μg Fe/gm) diet, with the transfusion of red cells being continued as usual. The chelator under investigation is administered daily at least 4 h after injection of the red cells. Urine and stool are collected daily for 5 days and their iron content determined by atomic absorption. When given parenterally, HBED induced twice the net urinary iron excretion of an equivalent dose of DFO and nearly three times the amount of stool iron (TABLE 1). Total iron excretion was more than two and a half times that due to DFO. Upon oral administration, HBED retained approximately 25% of its activity, being 70% as effective as DFO given parenterally. A prodrug, the dimethyl ester (dmHBED), was prepared in an effort to enhance gastrointestinal absorption. Hydrolysis of the ester linkages catalyzed by serum/tissue esterases would make HBED available for chelation of iron. When the prodrug was given orally, it was six times more effective than HBED itself and four times more effective than DFO given parenterally. In fact, oral administration of dmHBED proved to be 75% as effective as giving the same dose i.p. Neither HBED nor its dimethyl ester had any effect upon the excretion of calcium, magne-

TABLE 1. Net Iron Excretion Induced By Various Chelators in Hypertransfused Rats

Chelator (100 mg/kg/day)	Route[a]	*n*	Net Iron Excretion (μg/kg/day) Urine	Stool	Total	% DFO[b]
HBED	i.p.	6	178	1281	1459	265
	p.o.	12	49	335	384	70
dmHBED	i.p.	6	576	2571	3147	573
	p.o.	12	287	2123	2410	439
DMHP	i.p.	6	405	916	1321	241
	p.o.	6	321	647	968	176
PIH	i.p.	6	62	235	297	54
	p.o.	6	47	505	552	101
DFO	i.p.	54	84	465	549	
	p.o.	12	8	22	30	

[a]Route of administration was intraperitoneal injection (i.p.) or oral (p.o.).
[b]Excretion as a percent of that induced by DFO given i.p.

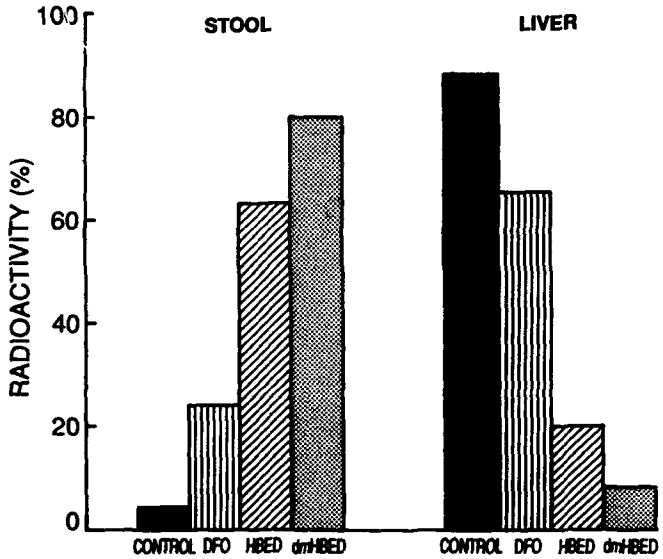

FIGURE 1. The percentage of radioactivity in the liver and accumulated stool of rats 6 days after labeling with ^{59}Fe-ferritin and subsequent intramuscular injection of desferrioxamine (DFO), *N,N'*-bis(*o*-hydroxybenzyl)ethylenediamine-*N,N'*-diacetic acid (HBED) or its dimethyl ester (dmHBED) at a dose of 200 mg/kg.

sium, copper, and zinc in these animals. For comparison, the relative effectiveness of PIH and DMHP is also shown in TABLE 1. The latter compound, being almost as effective as HBED when given i.p., proved to be more than twice as effective as HBED upon oral administration. Both PIH and DMHP were clearly inferior to dmHBED, however. The potential usefulness of HBED/dmHBED has subsequently been confirmed in other animal models of iron overload,[10,22,23] as well as in a variety of *in vitro* systems employing cultured Chang cells,[18] rabbit reticulocytes (P. Ponka, personal communication), and rat myocytes.[19]

Pharmacological studies of HBED and dmHBED undertaken by Hershko *et al.*[19,20] further emphasize the relative effectiveness of these compounds in comparison with DFO. The hepatic parenchymal cells of hypertransfused rats were labeled with ^{59}Fe-ferritin given intravenously.[24,25] One hour after labeling, a single injection of drug was administered intramuscularly to groups of four rats at a dose of 200 mg/kg. Urine and stool were collected daily for 6 days, after which the animals were sacrificed and residual radioactivity in their liver and spleen was measured, together with that in the accumulated urine and stool. None of the compounds caused any significant change in the ^{59}Fe content of urine or spleen. On the other hand, hepatic radioactivity declined in response to all three drugs. FIGURE 1 shows that 88% of the radioactivity in the livers of control animals remained after 6 days, with only 4% excreted spontaneously in the stool. In animals treated with DFO, 24% of the radioactivity appeared in the stool over the course of the 6 days. With equivalent doses of HBED and dmHBED, 63% and 80% of the respective amounts of total radioactivity appeared in the stool. As indicated in FIGURE 1, there was an inverse relationship between fecal iron excretion and residual hepatic radioactivity.

In a related study, ^{59}Fe-labeled heat-damaged rat erythrocytes were injected

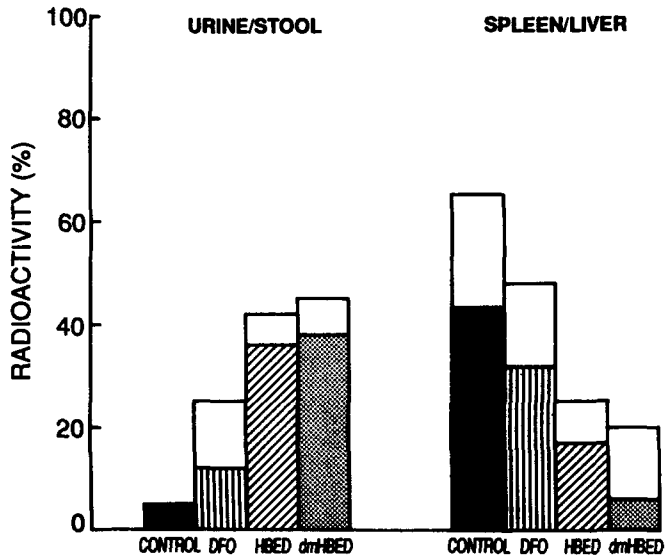

FIGURE 2. The percentage of radioactivity in the spleen, liver, and accumulated urine and stool of rats 6 days after infusion of ^{59}Fe-labeled heat-damaged rat erythrocytes and subsequent intramuscular injection of the indicated chelators at a dose of 200 mg/kg. The contributions of urinary and splenic radioactivity to the respective totals are indicated by the *open areas* at the top of each bar.

intravenously to label the reticuloendothelial system. Six days after labeling, 65% of the radioactivity in control animals remained in the liver and spleen and 6% was excreted spontaneously, roughly 90% of it in the stool (FIG. 2). In response to intramuscular injection of DFO (200 mg/kg), there was a fourfold increase in excretion of radioactivity, the percentage in the urine increasing from 0.1% to 12.8%. Administration of equivalent doses of HBED and dmHBED led to a sevenfold increase in excretion, with more than 80% of the radioactivity appearing in the stool. Here too there was an inverse relationship between the amount of radioactivity excreted and that which remained in the liver and spleen. That the ratio of splenic to hepatic radioactivity remained essentially constant following administration of HBED and DFO suggests that redistribution of iron from one tissue to another was not significant. The increased loss of hepatic radioactivity in response to dmHBED probably reflects differential tissue uptake of the diester rather than redistribution of iron. Residual radioactivity did not differ significantly from that in the case of HBED.

The situation regarding redistribution of iron was also addressed in a separate study. The ^{59}Fe-complexes of these chelators were prepared *in vitro* and then injected subcutaneously into normal rats.[14,23] Determination of cumulative excretion over 6 days revealed that 94% of the radioactivity initially bound to HBED was excreted compared to 84% in the case of the dimethyl ester and 75% for DFO. Thus, HBED can be expected to compete effectively with transferrin for iron, and, once bound, to cause preferential excretion of this iron rather than redistribution to other tissues. With most of the other iron chelators that have been evaluated, there was a much greater tendency toward redistribution.

The results of a dose-response study are shown in FIGURE 3. At low doses (25–50

mg/kg), HBED and dmHBED were ten to fifteen times more effective than DFO. As the dose increased, the relative effectiveness of HBED and dmHBED declined, due in large part to the decreased specific activity of residual liver iron stores. In these studies, urinary excretion of radioactivity was never more than 4% of that administered, dmHBED having the greatest effect at all doses. It remains to be seen how the degree of iron loading affects the relative bioefficiency of HBED and dmHBED.

The effectiveness and specificity of HBED having been established, an evaluation of toxicity was undertaken. The LD_{50} of both HBED and dmHBED exceeds 800 mg/kg when given either orally or parenterally to normal mice; no deaths occurred at this dose.[21] Chronic toxicity studies of HBED have now been completed in mice, rats, and dogs. Young male and female Swiss-Webster mice were given HBED i.p. and orally for 10 weeks at doses up to 200 mg/kg and 0.4% of the diet. When HBED was administered i.p., the growth of both the male and female mice was reduced relative to that of control animals, the effect being dose related. The urine and stool of these animals were slightly reddish, undoubtedly due to the iron complex of HBED. Otherwise, the animals appeared healthy. At sacrifice, gross pathological examination revealed no evidence of toxicity. Red and white cell counts were somewhat below normal in the treated males but above normal in the females. The changes were neither dose-related nor significant, however. A similar lack of toxicity was observed when HBED was given orally.

The most extensive studies were conducted in Sprague-Dawley rats. When HBED (50, 100 and 200 mg/kg) was administered i.p. to groups of six females, all of the animals grew at a normal rate. In the case of males, the growth of the drug-treated animals was approximately 15% less than that of the controls at all three doses. This effect was undoubtedly due in part to the fact that the controls were

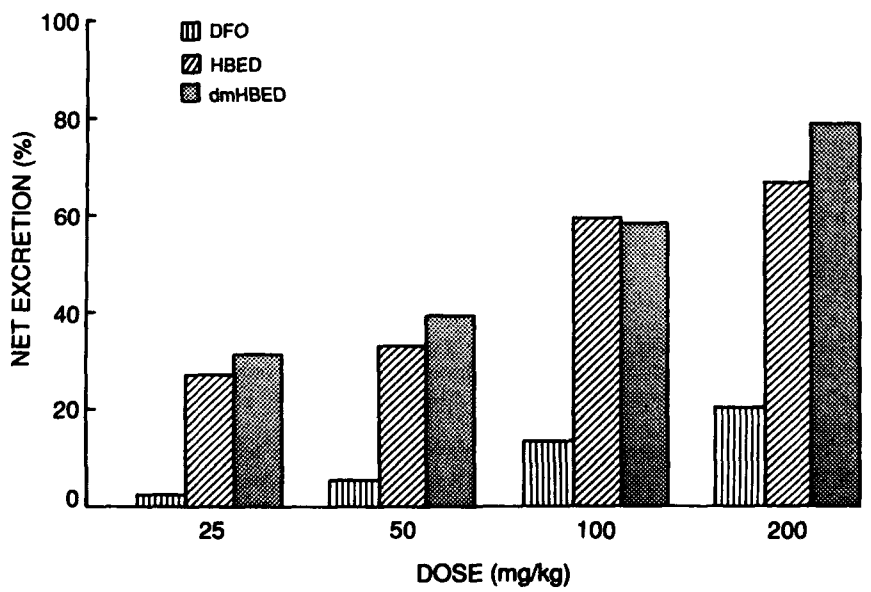

FIGURE 3. The net percentage of radioactivity in the accumulated urine and stool of rats 6 days after labeling with ^{59}Fe-ferritin and subsequent intramuscular injection of the indicated chelators at doses ranging from 25 to 200 mg/kg.

not sham injected. At sacrifice (12 weeks) none of the animals exhibited gross pathological abnormalities. The weight and appearance of major organs (liver, spleen, kidneys, adrenals, heart, lungs, testes/ovaries, and brain) were normal. Histopathological examination revealed no drug-related lesions. The white cell count was slightly decreased in females, with the opposite effect being seen in males. Serum chemistries were essentially normal in both groups of rats. Thus, HBED given i.p. at doses up to 200 mg/kg does not appear to result in significant toxicity.

Administered orally at doses up to 0.1% of the diet, HBED had no significant effect upon the weight of either male or female weanling rates. At doses of 0.2% and 0.4% of the diet, however, decreased growth rates were observed in both groups, 11% and 24%, respectively, in females; 17% and 32% in males. Half the animals in each group were sacrificed at approximately four weeks and the remainder after eight weeks. No gross or histopathological lesions were observed. While some

TABLE 2. Cellular Blood Profile for Male Sprague-Dawley Rats At 4 and 8 Weeks of Orally Administered HBED

	Hematological Values (mean ± SEM) for Indicated % of HBED in Diet				
Parameter[a]	0.0	0.05	0.1	0.2	0.4
4 Weeks					
WBC ($\times 10^9$/l)	6.8 ± 0.6	7.4 ± 0.3	7.5 ± 0.8	6.8 ± 2.3	5.6 ± 1.0
RBC ($\times 10^{12}$/l)	5.9 ± 0.2	5.9 ± 0.2	5.6 ± 0.1	5.0 ± 0.2[b]	4.6 ± 0.1[d]
Hb (g/dl)	13.9 ± 0.1	12.2 ± 0.4[b]	9.8 ± 0.3[e]	8.3 ± 0.4[e]	8.2 ± 0.2[e]
Hct (%)	39.8 ± 0.4	34.5 ± 1.0[c]	29.2 ± 1.0[e]	24.0 ± 1.2[e]	21.6 ± 0.5[e]
MCV (Fl)	67.3 ± 1.3	57.6 ± 1.2[b]	51.0 ± 1.0[e]	50.0 ± 1.7[d]	46.6 ± 0.3[e]
8 Weeks					
WBC ($\times 10^9$/l)	8.8 ± 2.1	9.9 ± 2.4	4.4 ± 1.1	7.5 ± 2.1	4.6 ± 0.8
RBC ($\times 10^{12}$/l)	6.9 ± 0.4	7.0 ± 0.1	6.8 ± 0.1	5.4 ± 0.1[b]	4.5 ± 0.4[b]
Hb (g/dl)	15.2 ± 0.2	14.2 ± 0.4	14.0 ± 0.3[b]	9.6 ± 0.4[e]	9.6 ± 0.5[e]
Hct (%)	39.5 ± 1.6	38.4 ± 1.8	36.3 ± 1.3	24.1 ± 0.6[e]	19.3 ± 0.2[e]
MCV (Fl)	55.3 ± 1.2	53.0 ± 2.1	51.7 ± 1.9	44.0 ± 0.5[d]	45.0 ± 0.5[d]

[a]WBC, white blood cell count; RBC, red blood cell count; Hb, hemoglobin; Hct, hematocrit; MCV, mean corpuscular volume.
[b]$p < .05$.
[c]$p < .01$.
[d]$p < .005$.
[e]$p < .001$.

differences in the weight of the major organs were noted, none appeared to be dose or time related. Undoubtedly they reflect the small number of animals ($n = 3$) in each group. In any case, no consistent differences were observed when comparing males and females.

The greatest changes upon administering HBED orally were observed in the red cell indices. At four weeks, the red cell count, hemoglobin level, hematocrit, and mean corpuscular volume of male rats were all significantly decreased in a dose-related manner (TABLE 2). In fact, reductions of 20% or more were observed with a dose of 0.4% HBED. After eight weeks these indices were still depressed in a dose-related manner, but the reductions were less dramatic, indicating a partial resolution of the situation. Female rats exhibited similar changes, but the magnitude of the changes was somewhat less than that seen in the case of the males. It is believed that these changes, as well as the decreased growth rates, reflect a functional iron deficiency due to HBED-mediated inhibition of dietary iron absorp-

tion. That a hemolytic process was not responsible is indicated by the low-to-normal levels of splenic, hepatic, and marrow iron observed. These findings in no way contraindicate the use of HBED in patients with transfusional iron overload.

The serum chemistries of the drug-fed rats also exhibited a few abnormalities, but none appeared to be dose or time related. For instance, when compared to control values, the blood urea nitrogen (BUN) of the females was somewhat reduced at all doses after four weeks. At eight weeks, however, the values were normal. Drug-treated males, on the other hand, exhibited a normal BUN at four weeks but elevated values at all doses after eight weeks, although none of the increases were statistically significant. The total bilirubin levels of the females were slightly low at both time intervals and in the males mildly elevated at four weeks but normal at eight weeks. Liver function tests also showed some abnormalities. At eight weeks, the serum glutamic-pyruvic transaminase (SGPT) was decreased in females but elevated in males. The serum glutamic-oxaloacetic transaminase (SGOT) was slightly elevated in males at both times but not in females. Again, few of the changes observed were significant and none appeared to be dose-related. Finally, urinalyses were essentially normal, there being a slight shift to higher pH and lower specific gravity with increasing dose of drug.

The toxicity of HBED was also evaluated in three male and three female beagle dogs at a dose of 100 mg/kg, an additional three females serving as controls. The compound was administered orally in gelatin capsules once each day for six weeks. The drug-treated animals all grew normally and experienced no known side effects. Their appetite was good, with no vomiting or diarrhea observed. Weekly serum chemistries and cellular blood profiles did not reveal any significant changes. An ophthalmologic examination after six weeks revealed no drug-related abnormalities. Likewise, there were no neurological deficits noted. At sacrifice, no gross or histopathological lesions were seen. The weight and appearance of the major organs were normal.

The relative safety and efficacy of HBED having been established, we are now planning to evaluate this chelator in thalassemia patients. Our primary goal will be to compare the excretion of iron induced by comparable doses of HBED and DFO. Each drug will be administered for seven days with an appropriate recovery period between drug treatments. In the meantime, studies with dmHBED are continuing. Once a cost-effective method of synthesis for this prodrug has been developed, it too will be evaluated in humans if it proves to be nontoxic after extensive animal studies. All of the evidence accumulated thus far suggests that HBED may be a useful oral chelator, with dmHBED holding forth even more promise.

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Evaluation of the Oral Iron Chelator 1,2-Dimethyl-3-hydroxypyrid-4-one (L1) in Iron-Loaded Patients[a]

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Despite the problems associated with its chronic administration to patients with Cooley's anemia, parenteral deferoxamine (DFO) remains the mainstay of iron chelation therapy.[1] Intensive administration of this drug is associated with serious neurotoxicity and considerable cost. However, the most problematic aspect of long-term therapy with DFO is the requirement for subcutaneous infusion using a battery-powered pump. Consequently, as early as 10 years of age, patient compliance with subcutaneous DFO becomes erratic, and it declines further during the teenage years, at the time when transfusional iron accumulation is accelerated.[2]

An increasing body of evidence, some of which has been presented at the meeting on which this volume is based, indicates that iron chelation, although problematic, reduces the morbidity and mortality associated with iron overload. Regular DFO therapy removes hepatic iron[3-5] and prevents hepatic fibrosis[5] in iron-loaded patients. Survival,[6] and in some patients linear growth[7] and sexual maturation,[7,8] may also improve. Reversal of iron-related cardiac dysfunction has been less convincingly demonstrated. In some patients with Cooley's anemia, clinical improvement in cardiac function and unexpectedly prolonged survival have followed intensification of DFO therapy.[9,10] In other patients, progressive cardiac deterioration has not been altered.[10,11] It appears that the key to the preservation of organ function in transfusion-dependent patients may be the prevention of accumulation of iron in body tissues, including the heart.[1]

The failure of many patients with Cooley's anemia to comply with subcutaneous DFO therapy is a major obstacle to prevention of iron-related morbidity and

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mortality.[12] Oral chelation not only has the potential to make long-term therapy more acceptable to the patient, but it may also increase efficacy by providing a continuous supply of circulating drug.[13] A safe, inexpensive, orally available chelating agent would also make therapy available to the thousands of Cooley's anemia patients worldwide for whom chelation is presently unavailable and who die of iron overload. During the last ten years, many oral chelators have been tested in rodent or primate models of iron overload. Some are discussed elsewhere in this volume; a few have been the subject of human trials.[14–17]

HYDROXYPYRIDONE CHELATORS

1,2-dimethyl-3-hydroxypyrid-4-one, or L1 in the terminology used by its first clinical investigators,[14,15] is one of several hydroxypyridone chelators which have been evaluated in cell culture,[18] animal studies,[19–21] and a small number of patients.[14,15] Hydroxypyridones are neutral bidentate ligands with a high specificity for ferric iron. The stability constant of their iron complexes ($\log K_a = 37$) is six orders of magnitude higher than that of DFO. In animal studies, the source of chelated iron was found to be mainly hepatocellular. When L1 was administered to iron-loaded patients, it resulted in urinary iron excretion similar to that obtained by equivalent doses of parenteral DFO.[14,15] However, baseline fecal iron excretion was not measured in these studies, and patients in the control and treatment groups were not matched for hemoglobin levels. Since DFO has been shown previously to induce fecal iron excretion at levels of 10–16% of total iron excretion in a patient with a low hemoglobin level and of 22–52% in a different patient with a high hemoglobin level,[22] the total evaluation of a new chelator necessitates careful quantification of fecal iron excretion and attention to the hemoglobin level at the time of iron excretion.

A cohort of our iron-overloaded patients with Cooley's anemia do not achieve net negative iron balance with DFO, either because significant neurotoxicity limits their dose, or because of poor compliance with the regimen of subcutaneous DFO. They are thus at high risk of organ toxicity and death from iron overload. Earlier trials of L1 in iron-loaded patients in the United Kingdom have demonstrated its short-term efficacy in a small number of patients.[14,15] Accordingly, we administered L1 to patients with transfusional iron overload in whom DFO therapy was failing. The results demonstrate that in the short-term, L1 is as effective as DFO in promoting iron excretion and is well tolerated by these patients.

PATIENTS AND METHODS

Twenty-six transfusion-dependent patients with a mean age of 22 years (range, 8–49 years) were admitted to the hospital for supervised administration of L1. Twenty-four patients had Cooley's anemia, and two had Diamond-Blackfan anemia. Fifteen patients were poorly compliant with nightly subcutaneous DFO for at least two years (defined as $<80\%$ of the prescribed amount of drug taken), and 6 patients had refused subcutaneous DFO completely. These patients were considered at risk for accelerated iron loading. An additional five patients had DFO-induced neurotoxicity[23] that limited the administered dose of DFO, thereby precluding net negative iron balance. Ten further patients who were eligible for the study refused to participate.

All patients had evidence of moderate-to-severe iron overload, with serum

ferritin levels of 2579 ± 1670 (range, 705–7645) μg/l and transferrin saturations of 96 ± 5 (86–100) %. Liver dysfunction was present in 17 patients, whose aspartate aminotransferase (ASAT) values were 73 ± 82 (17–377) U/l. Nine patients had insulin-dependent diabetes mellitus and one showed evidence of hypothyroidism and hypoparathyroidism. Flat responses to LHRH stimulation testing were present in 13 patients. Eight patients required therapy with digoxin and diuretics for iron-related cardiac dysfunction. The mean transfusional iron exposure (grams of iron transfused since the initial transfusion, per kilogram of present body weight) was 2.1 ± 0.3. Mean total DFO administered (estimated grams of DFO since initiating chelation therapy, per kilogram present body weight) was 89.4 ± 31.9.

Permission for Study

The oral use of L1 has been reported in human studies in the United Kingdom.[14,15] The compound is not available commercially. However, on the basis of the animal studies generated by other investigators,[19–21] application was made to the Health Protection Branch, Health and Welfare Canada, for permission to produce and administer L1 to patients with severe transfusional iron overload who do not have DFO as a therapeutic option. These patients, either because of serious DFO-related neurotoxicity or a long history of poor compliance with regular DFO therapy, are destined to die of iron overload. In September, 1988, permission was obtained for a pilot study (File No. 9427-H1117-41C, HPB, Ottawa, Canada). The study was approved by The Hospital for Sick Children's Human Subject Review Committee. Written informed consent was obtained from each patient, or from a parent in cases of children younger than 16 years, prior to entry into the study.

Preparation of 1,2-Dimethyl-3-hydroxypyrid-4-one (L1)

Synthesis of L1 was carried out according to previously published methods[24] by the direct reaction in aqueous solution of methylamine and maltol. The brown material so obtained was purified by four recrystallizations from water, giving fine white needles in a yield of about 50% based on the amount of maltol originally employed. Purity of the sample was established by melting point, elemental analysis, 400-MHz ^{1}H-NMR, infrared and mass spectroscopy, and thin layer chromatography on silica gel with chloroform/methanol or a reverse-phase C_{18} plate with methanol/water. Capsulization of L1 in 300 mg quantities was performed in the Department of Pharmacy at The Hospital for Sick Children, Toronto.

Study Protocol

Randomized Crossover Comparison of Urinary Iron Excretion with L1 and DFO Treatment

The protocol for the crossover study is shown in FIGURE 1. Twenty patients were admitted following their monthly transfusions. Patients who took DFO at home were instructed to discontinue infusions of the drug 72 h prior to transfusion. A low-iron diet (9.2 ± 1.6 mg/day, mean ± SD) was started on admission. The following day, a collection of urine for baseline iron quantification was begun, with all urine voided into acid-washed plastic containers, under nursing supervision. Blood samples were

obtained at baseline and on days 1, 3, and 5 for cell counts, white blood cell differential counts, and assays of serum electrolytes, blood urea nitrogen and creatinine, total protein, albumin, ASAT, alanine aminotransferase (ALAT), calcium, phosphorus, magnesium, copper, and zinc. DFO concentration was measured in the first serum sample. Twenty-four hours after admission, each patient began randomized therapy with either L1 or DFO, both at a dose of 50 mg/kg/day. L1 was given in divided doses at 0800, 1600 and 2400 hours, at least one hour before or after ingestion of food or liquids. DFO was infused over 12 h. Each drug was administered on days 2, 3 and 4; and all voided urine was collected in 24-h aliquots and analyzed for iron. Patients were discharged on the morning of day 6. Following the next blood transfusion 3–4 weeks later, each patient was readmitted and received the alternate drug therapy under the same protocol. Each patient therefore served as his or her own control in this phase of the study. This protocol ensured that each patient was studied at a similar hematocrit during both treatments, an important consideration since urinary and fecal iron excretion may vary in patients with homozygous β-thalassemia, depending on the hemoglobin level.[22]

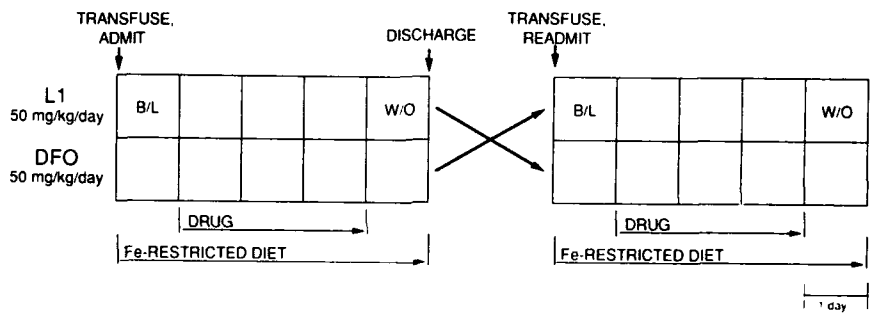

FIGURE 1. Protocol for randomized crossover comparison of L1 and DFO. Following transfusion, patients were begun on an iron-restricted diet, and urine was collected daily during baseline (B/L) and washout (W/O) periods, as well as during the 3 days of drug treatment. Following the next transfusion (3–4 weeks later), the procedure was repeated with the alternate drug. Choice of first drug was random.

Five patients who participated in the crossover study were readmitted to the hospital immediately following transfusion at a later date for administration of L1 at a dose of 75 mg/kg/day using the above protocol, to determine the impact on urinary iron excretion of an increase in the dose of L1.

Total Iron Excretion Studies with L1

Six patients were admitted to evaluate total iron excretion (fecal and urinary) during L1 therapy (75 mg/kg/day). The protocol for this study is shown in FIGURE 2. Throughout this study, patients were maintained on the low-iron diet. On admission, each patient received 10 μCi of $^{51}CrCl_3$ orally in 5 ml of distilled water. Stool was then counted for radioactivity to document excretion of ^{51}Cr. After 95% of the label had been recovered in stool (requiring 3–5 days in all patients), all urine and stool were saved in acid-washed containers for the next 72 h (B/L, FIG. 2), in 24-h (urine) and 72-h (stool) collections. When these collections were completed, a second dose of 10

μCi of $^{51}CrCl_3$ was administered 3 h following the first dose of L1. Administration of L1 then continued at 8-h intervals for the remainder of the study. When 95% of the second dose of ^{51}Cr was recovered in stool, three 72-h collections (I, II, III, FIG. 2) of all stool and daily collections of urine were analyzed for iron. With the use of $^{51}CrCl_3$, it can be expected that, upon start of the baseline fecal iron collection, all iron consumed prior to the institution of iron restriction had passed out of the gastrointestinal tract. Similarly, the collections for fecal iron during L1 therapy were begun only after stool formed before the first ingestion of L1 had been excreted.[25]

Analyses

Urine iron was determined by direct atomic absorption spectrometry of a diluted sample, using a Varian Spectra AA-10 atomic absorption spectrophotometer (Varian Techtron, Australia). The analyzer was calibrated using standard iron solutions

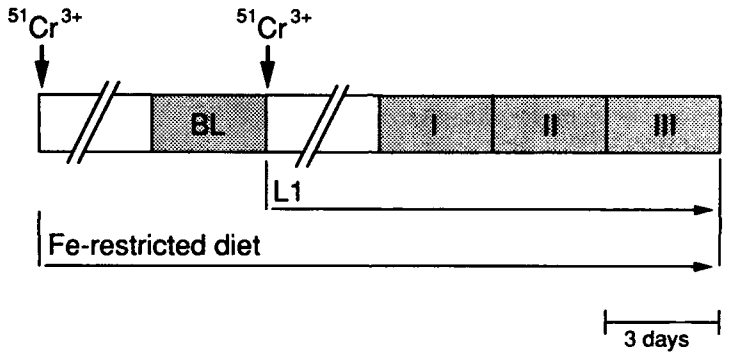

FIGURE 2. Protocol for total iron excretion study. Patients underwent one 72-h stool collection for baseline (B/L) iron determination and three 72-h collections (I, II, III) for fecal iron measurement during L1 treatment. All urine voided during B/L, I, II, and III was also analyzed for iron. The period of time for elimination of analyzed $^{51}CrCl_3$ in stool varied from 3 to 5 days.

prepared from BDH Analytic Grade iron standard. Stool was processed for iron analysis by a method based on that of Hilliard and Smith.[26] Stool was liquified by shaking with an equal volume of water in the tared plastic collection containers, and triplicate aliquots of 1–3 g were digested by gentle heating in 10 ml of concentrated HNO_3 (GR; Merck, Darmstadt). After volume reduction, the procedure was repeated three times using 10 ml of HNO_3:H_2O_2 (1:1). The final concentrate was diluted to 25 ml with water. Iron was measured by flame atomic absorption spectrometry (Perkin-Elmer 306 spectrometer). Method blanks were prepared with each batch of samples, and measurements were shown to be free from matrix effects by addition of standard. DFO was measured at baseline by high pressure liquid chromatography (HPLC)[27] to ensure that washout of the drug from previous therapy was complete. The detection limit was 1 μg/ml.

The quantitative measures of iron excretion before and during chelation therapy were compared by paired Student's *t* test. Correlation between parameters was

tested by linear least squares regression analysis. Values are expressed as mean ± SD.

RESULTS

Overall, urinary iron excretion induced by the administration of DFO was superior to that induced by L1, although the two drugs induced comparable urinary iron excretion in several patients at doses of 50 mg/kg/day. L1 was then administered at an increased dose of 75 mg/kg/day to five patients. In these patients, urinary iron excretion was comparable or superior to that achieved with DFO administration.

Results of the total iron excretion study illustrate several important points. First, it should be noted that collections to quantify fecal iron excretion induced by a chelator cannot be carried out without establishing careful measurements of baseline fecal iron. This can be effectively accomplished with the use of $^{51}CrCl_3$, which was administered orally to each patient at the start of the balance study. $^{51}CrCl_3$ is not absorbed, and it therefore serves as a marker of stool transit. Most of our patients required at least five days to excrete 95% of the label before a 72-h baseline (drug-free) collection for fecal iron was begun. Of course, stool "washout" times reflect individual variability, aggravated in the present study by the constipation experienced by many of the patients on the low-iron hospital diet. Stool softeners and sorbitol-containing chewing gum were needed in all cases. These difficulties were experienced following each administration of $^{51}CrCl_3$, and they resulted in the prolongation of the hospital stay beyond the original expectations of both physicians and patients.

A typical patient illustrates these principles. A twenty-year-old Saudi Arabian male with Cooley's anemia developed severe auditory toxicity with intensive use of subcutaneous DFO in 1985. Repeated attempts to increase his dose of DFO to achieve negative iron balance were thwarted by worsening high frequency sensorineural hearing loss. He has refused the assistance of a hearing aid, despite clinical hearing difficulties. His serum ferritin was over 2000 μg/l during the year prior to his iron-balance study. He was therefore offered the option of experimental therapy with L1. He required five days to excrete over 95% of the stool marker. He then began baseline fecal collection and was found to excrete 34.3 ± 4.2 mg iron in the stool over 72 h, whereupon the second dose of $^{51}CrCl_3$ was given at the onset of L1 therapy. A further five days was again required for full ^{51}Cr excretion. A collection begun immediately thereafter to assess the effects of L1 on fecal iron elimination documented a mean of 11.4 mg fecal iron over baseline per 72 h.

DISCUSSION

Our study demonstrates that, in many patients, urinary iron excretion achieved with L1 at a dose of 50 mg/kg/day is comparable to that induced by parenteral infusion of standard doses of DFO. In those on L1 with lower iron excretion values, an increase in the dose of L1 to 75 mg/kg/day may result in iron output comparable to that achieved with DFO.

A significant decrease in urinary iron excretion is known to occur with administration of DFO at a high hemoglobin level, associated with less ineffective erythropoiesis, relative to that achieved at a lower hemoglobin level, associated with active erythropoiesis.[22] On entry into the present study, all patients had a high hemoglobin

level, and therefore their monthly urinary iron excretion with L1 therapy might be greater than that observed in this short-term study.

In the evaluation of any new iron chelator, it is necessary to assess not only urinary but also fecal iron excretion induced by the drug under study. Pippard *et al.*[22] reported total iron balance studies in two patients with Cooley's anemia treated with subcutaneous DFO. Five heavily iron-loaded patients in the present study showed comparable urinary excretion to those in Pippard's study, who excreted a mean of 13% of their total iron output into stool at a low mean hemoglobin (8.3 g/dl) and 37% at a higher (11.7 g/dl) level. Both patients had plasma ferritin levels in excess of 5000 μg/l.[22] In another study of 27 patients,[28] other investigators showed fecal iron excretion in response to administration of subcutaneous DFO to be highly variable (32–62%). With intravenous DFO, fecal iron varied between 6 and 84% of total iron excretion.[29] Some patients actually excreted less fecal iron after intravenous administration than they did on subcutaneous DFO at half the dose. These studies are in contrast to those of Pippard *et al.*,[22] who noted that the proportion of iron excreted in stool was much greater (49–79% of total) when DFO was infused intravenously. Before these studies can be compared with each other and with the present report, it is necessary to know the criteria used to measure fecal excretion in each case. As pointed out here, it is necessary to demonstrate with the use of a stool transit marker that true baseline and treatment levels are being observed. We have found that several days may be required for adequate elimination of the $^{51}CrCl_3$ marker.

It should be pointed out that declines in hemoglobin, white cell count, and platelet count were recently reported to have occurred in laboratory mice after 60 days of intraperitoneal administration of L1 at a dose of 200 mg/kg/day.[30] A second group of investigators administered similar doses to rats for 90 days and observed a fall in hemoglobin and white cell count but not platelet count. No changes in hematological indices were reported when 60 mg/kg doses of L1 were administered intraperitoneally to rats for 36 days.[31] The doses of L1 used in the present study were substantially lower than those reported to cause myelotoxicity in rodents. The oral bioavailability of L1 is only about one-third of its parenteral value.[13] Hence, at the oral doses administered to patients in this study, serum drug levels are probably manyfold lower than those achieved after intraperitoneal administration of L1 in animal studies. However, one patient with a primary marrow disorder (Diamond-Blackfan anemia) and borderline neutropenia developed agranulocytosis while receiving L1 for iron overload.[32] The cause of marrow failure in this patient is not clear.

The development of a safe, orally available chelator is of paramount importance to the millions of iron-loaded patients worldwide. Although subcutaneous DFO has been demonstrated to be a highly effective chelating agent, it cannot be utilized by all Cooley's anemia patients. L1 is an efficacious oral chelator in the short term and is well tolerated by patients for whom DFO therapy is failing either because of neurotoxicity or poor compliance. Nevertheless, it should be stressed that the use of this drug as a therapeutic alternative to subcutaneous DFO cannot yet be recommended but must await long term toxicity testing in animals.

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A Comparative Evaluation of Iron Clearance Models

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INTRODUCTION

The utilization of iron in primates is characterized by a highly efficient recycling process,[1-4] and there is no specific mechanism for its elimination. Once "excess iron"[5-7] is introduced it remains in the system, ultimately leading to peroxidative tissue damage. In several iron-overload states the only solution is to maintain patients on chelation therapy. Unfortunately, the current methodology, desferrioxamine infusion, is not satisfactory as many individuals must be maintained on this regimen for most of their natural lives. It is therefore not surprising that one of the major difficulties in the treatment is associated with patient compliance. The second issue is associated with the absence of the technology required for the desferrioxamine treatment in Third World countries. Identification of an orally effective iron chelator would solve both of these problems.[8]

Numerous compounds are already available which bind iron very tightly, and investigators have identified the structural parameters necessary for future drug design.[9-10] Many of these ligands have been effective at removing iron in cell culture models[11] and have been promoted to the next level of evaluation, the rodent model.[12] In fewer cases, data from rodent studies have led to toxicity screening and then to human trials. Numerous compounds which appeared outstanding in both of these screens failed at a clinical level. It is not clear that the rat model is the best indicator of how ligands can be expected to work in humans.

More recently, investigators have considered the *Cebus* monkey model[13] as an intermediate screen for evaluating iron chelators prior to human studies. However, the initial work indicated problems with background noise; iron-clearance levels were far in excess of theoretical possibility. It is the purpose of the current investigation (1) to address the problems associated with fecal iron noise levels, (2) to compare and contrast the bile duct–cannulated, non–iron-overloaded rat model with an iron-overloaded cebus monkey model, and (3) to determine what correlation if any exists between the two systems.

EXPERIMENTAL MATERIALS AND METHODS

Materials

DFO (desferrioxamine B) was supplied by Ciba-Geigy Ltd., Basel Switzerland, in the form of the methanesulfonate salt (trade name: Desferal). The sodium salt DFT (desferrithiocin, CGP23841A) was obtained from the same source. The pyridoxal isonicotinoyl hydrazone (PIH) analogue 1-[*N*-ethoxycarbonylmethylpyridoxylidenium]-2-[2′-pyrimidyl]-hydrazone (bromide form: CGP43902A), prepared according to the procedure described in example 5 of Ref. 14, was kindly provided by the YISSUM Research and Development Company, Jerusalem, Israel. Sprague-Dawley rats were purchased from Charles River, Wilmington, Massachusetts. *Cebus apella* monkeys were obtained from World Wide Primates, Miami, Florida. All reagents and standard iron solutions were obtained from Aldrich Chemical Co., Milwaukee, Wisconsin. Nalgene metabolic cages, rat jackets, and fluid swivels were purchased from Harvard Bioscience, South Natick, Massachusetts. Intramedic polyethylene tubing PE-50 was obtained from Fisher Scientific, Pittsburgh, Pennsylvania. Atomic absorption (AA) measurements were made on a Perkin-Elmer model 5100 PC. Ultrapure salts were obtained from Alpha Products. Imferon, iron dextran, was obtained from Fisons.

Atomic Absorption Iron Detection

Samples are analyzed on a Perkin-Elmer 5100 PC atomic absorption spectrophotometer fitted with a model AS-51 autosampler, using a quartz sampling probe. The urine and bile samples are analyzed at 248.3 nm (slit 0.20 nm), and fecal samples at the less sensitive wavelength of 271.9 nm (slit 0.20 nm) with appropriate linear calibration of 0–5 ppm and 0–15 ppm, respectively. Representative samples are checked by the method of addition.

A 10-ml sample of urine is acidified with 500 μl of concentrated, low-iron nitric acid. Any particulates are separated by centrifugation, and the sample is analyzed directly by flame AA. The HNO_3-digested fecal samples are centrifuged if necessary and analyzed.

Drug Preparation

Desferrioxamine is administered subcutaneously to rats in deionized distilled water. Desferrithiocin and the PIH analogue are prepared in 60% water, 40% solubilizing agent and administered orally. Chelators are given to monkeys in low-iron gelatin capsules with the aid of a pilling gun. Desferrioxamine is administered to monkeys subcutaneously in sterile water for injection.

Non–Iron-Overloaded, Bile Duct–Cannulated Rat

Bile Duct Cannulation

Male Sprague-Dawley rats averaging 450 g are housed in Nalgene plastic metabolic cages during the experimental period and are given free access to food and water. The animals are anesthetized using sodium pentobarbital (50 mg/kg), given

intraperitoneally (i.p.). The bile duct is cannulated using 22-gauge polyethylene tubing (Intramedic, P.E.), about 1 cm from the duodenum. The cannula is inserted about 2 cm into the duct, and once bile flow is established, the cannula is tied snugly in place. A skin tunneling needle is inserted from the shoulder area around to the abdominal incision. The cannula is threaded through the needle until it emerges from the shoulder opening.

The cannula is passed from the rat to the swivel inside a metal torque-transmitting tether, which is attached to a rodent jacket around the animal's chest. The cannula is directed from the rat to a Gilson micro fraction collector by a fluid swivel mounted above the metabolic cage. This system allows the animal to move freely in the cage while continuous bile samples are being collected.

Bile and Urine Samples

Bile samples are collected in plastic disposable test tubes at 3-h intervals for 24 h. Urine samples are collected in disposable plastic tubes for 24 h. Iron output is calculated in terms of μg Fe excreted per kilogram of rat weight. The measurement of iron levels is performed using both a colorimetric (bathophenanthroline disulfonic acid) and the flame AA method. Rat urine (2.5 ml) is diluted to 7.5 ml with 6% low-Fe nitric acid, heated (65°C., 4 h), and filtered before analysis by flame AA. Bile samples are handled in the same manner as the urine specimens except that filtration is unnecessary.

Primates

Cages

During the evaluation of various iron chelators, the animals are moved from normal primate cages to specially constructed metabolic cages. These metabolic cages (FIG 1) are fabricated from Plexiglass and are approximately 4 ft. × 4 ft. × 3 ft. in size. Five of the faces have 1-in. diameter holes every 2 in. The bottom of the cage consists of a floor made of 1-in. Plexiglass rods spaced every ¼ in. apart. Beneath the floor is a plastic screen, which separates the feces from the urine. The very bottom of the cage is appropriately tilted so that the urine flows out through a spigot where it is collected in a bottle. Furthermore, the back of the cage is equipped with a second ventilated wall so that the monkeys can be squeezed to the front of the cage in order to be given injections when necessary. The animals are housed in these cages seven days prior to exposure to the chelator of interest and are maintained on a low-iron diet for this time and during the course of the chelator treatment.

Iron Loading

After intramuscular (i.m.) anesthesia with Ketamine, an intravenous infusion is started in a leg vein. The iron dextran is added to approximately 90 ml of sterile normal saline and administered to the animals at a dose of 200–300 mg/kg. The iron is infused over 45–60 min. Two to 3 infusions separated by 10–14 days are necessary to load the monkeys to a level of 500 mg/kg of iron. This brings the serum transferrin iron saturation to 70–80%. Liver biopsies were done on two animals under ultrasound control with a skinny needle. The anesthesia for the biopsy procedure is the

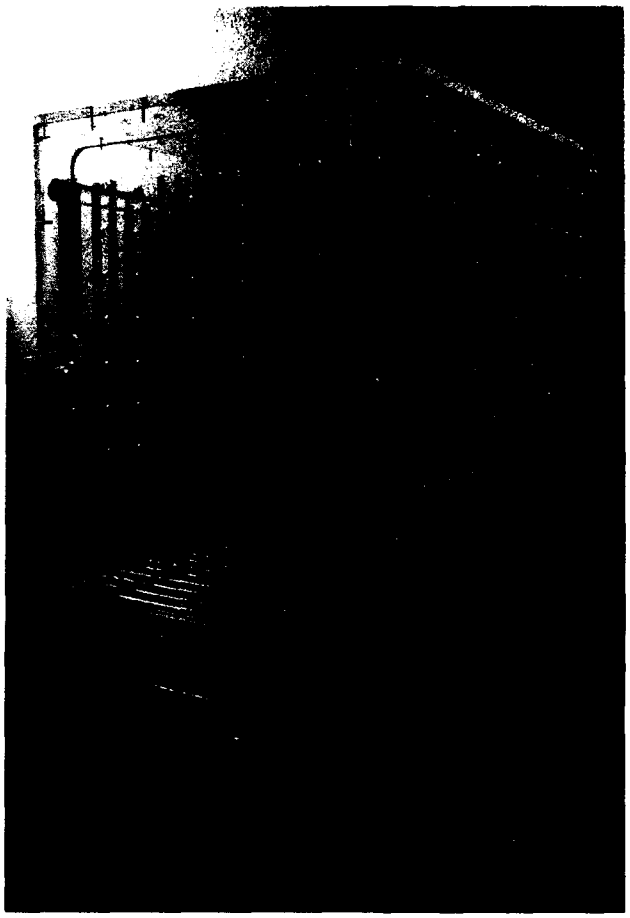


FIGURE 1. Plexiglass metabolic primate cage. The cage consists of three essential segments: a 4-× 4-× 3-foot living space, an animal-squeezing device at the back of the cage, and a collection tray which separates urine from feces.

same as that for the iron loading. The biopsy slides are fixed and then stained with hematoxylin-eosin, as well as with the iron stain (Prussian blue).

Low-Iron Diet

A low-iron liquid diet is prepared by first mixing the following ingredients: casein, 180 g; sucrose, 194 g; dextrin, 194 g; dextrose, 194 g; cellulose fiber, 90 g; vitamin mix, 5 g; methionine, 5 g; flavoring, 2 g; choline chloride, 2 g; and cholesterol, 1 g. A solution of the following liquids is added in portions with mixing: corn oil, 45 g; coconut oil, 45 g; and soy lecithin, 20 g. Finally, the following ultrapure grade salts are added to 1,350 ml of distilled deionized water: sodium chloride, 5.68 g; manga-

nese sulfate, 0.04 g; calcium carbonate, 10.16 g; potassium dihydrogen phosphate, 11.93 g; and magnesium sulfate, 3.45 g.

Fecal and Urine Samples

Fecal and urine samples were collected at 24-h intervals. Fecal samples are assayed for the presence of occult blood, weighed and mixed with a known volume of distilled deionized water, and autoclaved for 30 min. This mixture is then freeze-dried, and a portion of the powder is mixed with low-iron nitric acid and refluxed for 12 h. Once particulate matter in the digested samples is removed by centrifugation, iron concentrations are determined by flame AA. Monkey urine samples are acidified and reconstituted to initial volume after sterilization if necessary.

Hematologic Studies

Baseline blood values are obtained prior to iron overloading in all monkeys. These tests include hematocrit, white blood cell count, red blood cell indices, blood smear, reticulocyte count, and measurement of serum iron and total iron-binding capacity. Kidney and liver function studies including SGOT, SGPT, alkaline phosphatase, bilirubin (both direct and indirect), BUN, and creatinine are also evaluated. An initial blood sample is drawn seven days prior to chelator evaluation and another at 4–5 days after drug administration.

RESULTS

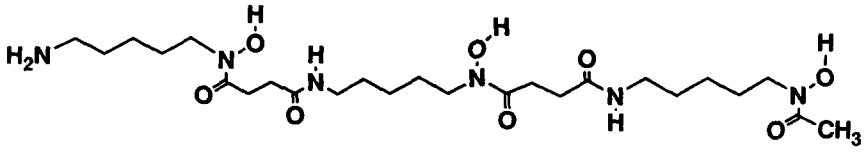
Chelator-induced Iron Clearance in Rodents

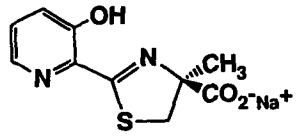
Three iron chelators were chosen (FIG. 2) for evaluation: desferrioxamine, a hydroxamate; desferrithiocin, a natural product thiazoline ring–containing ligand; and CGP43902B, a pyridoxyl hydrazone derivative. Subcutaneously administered desferrioxamine is used as a positive control, while orally administered desferrioxamine is used as a negative control. Each drug is given on an equimolar scale, 150 μmol/kg. The ratio of iron-binding equivalents for the series desferrioxamine: desferrithiocin:pyridoxalhydrazone is 1:2:2, chelator:Fe. The data comparing the three compounds are plotted in FIGURE 3 as cumulative bile iron output per unit rat weight. In TABLE 1, the data are presented as urinary, biliary, and total iron output per 24 h and indicate the efficiency of each compound. The calculated efficiency values are based on previous stoichiometry studies. The order of activity is desferrithiocin $\gg$ desferrioxamine $>$ PIH.

Primates

Monkey Housing

All animals are housed in separate cages in a single large room and moved to the metabolic cages in the same room seven days prior to administration of the drug. The





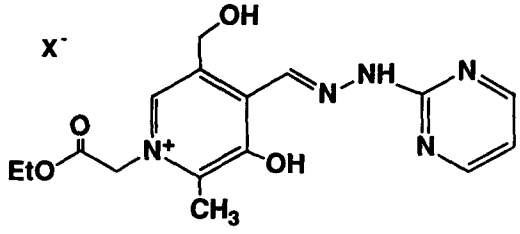


FIGURE 2. Structural formulas of desferrioxamine (DFO), desferrithiocin (DFT), and a PIH analogue (CGP43902B).

animals do not exhibit any signs of apprehension on being transferred to the metabolic cages, a key issue in observing drug-induced behavioral changes.

Iron Loading

Intravenous injection of Imferon do not result in any untoward effects in the animals. The final level of iron in each animal is calculated to be approximately 500 mg/kg. The iron loading in the monkeys was monitored in two animals by performing liver biopsies. The time between the intravenous iron loading and the biopsy was at least twelve months. The iron stain showed iron in both the hepatocytes and the reticuloendothelial (R.E.) cells. The biopsy from the animal that was loaded with 300 mg/kg showed less iron staining than the one loaded with 500 mg/kg.

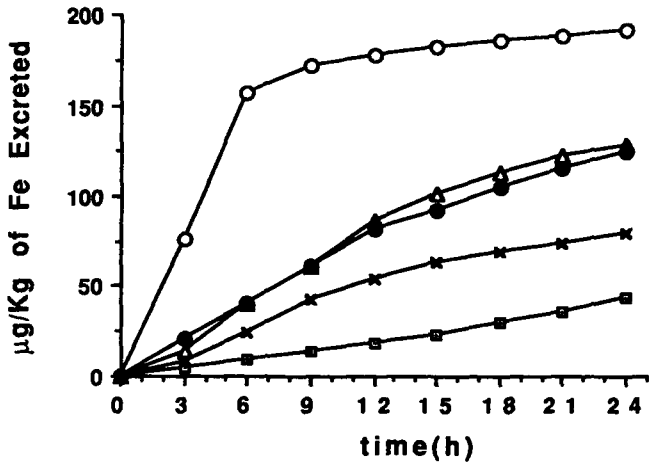


FIGURE 3. Cumulative biliary iron output (µg/kg) of bile duct–cannulated rats. Drugs were administered at 150 µmol/kg. (□) Control (oral), (△) desferrithiocin (oral), (○) desferrioxamine (subcutaneous), (●) PIH analogue (oral), and (×) desferrioxamine (oral).

Hematological Screens

All blood counts (CBC) and kidney and liver profiles except ferritin levels fall within the accepted normal range of the human values. Monkey ferritin could not be measured by utilizing the human anti-ferritin antibody assay.

Monkey Diet

The iron in Purina Monkey Chow is 150 ppm and is concentrated in the stool to 450 ppm. However, it is clear that maintenance of the animals on a low-iron diet for

TABLE 1. Effect of Iron-Chelating Drugs on 24-h Cumulative Iron Excretion in the Urine and Bile of Non–Iron-Overloaded, Bile Duct–Cannulated Rats

	Iron Excretion[a]					
	DFO (150 µmol/kg, s.c.) $n = 5$		DFT (150 µmol/kg, p.o.) $n = 4$		PIH Analogue (150 µmol/kg, p.o.) $n = 5$	
Measurement	mg/kg	%[b]	mg/kg	%[b]	mg/kg	%[b]
Theoretical total	8.37	100	4.19	100	4.19	100
Experimental total	0.28 ± 0.05	3.3	0.15 ± 0.02	3.5	0.14 ± 0.02	3.4
In urine	0.09 ± 0.03	30.5	0.02 ± 0.003	12.3	0.02 ± 0.005	13.3
In bile	0.19 ± 0.04	69.5	0.13 ± 0.02	87.7	0.12 ± 0.02	86.7

[a]Route of administration (s.c., subcutaneous; p.o., oral) and number of animals studied (n) are indicated for treatment with DFO (desferrioxamine B), DFT (desferrithiocin), and the PIH (pyridoxal isonicotinoyl hydrazone) analogue CGP43902B. The molecular weights and ligand: Fe binding ratios, respectively, for these drugs are (DFO) 656, 1:1; (DFT) 260, 2:1; and (PIH analogue) 442, 2:1.

[b]Values for urine and bile are calculated as % of corresponding total experimental value.

four days is ample time to achieve a new baseline. The initial low-iron diet evaluated, Bio-Serv wafers, contains 25–35 ppm iron. The metal is concentrated to 400 ppm in the stool. This diet results in tremendous variation in iron concentration in the stool as well as in total iron output (FIG. 4). Although it is sometimes possible to recognize iron spiking induced by excellent iron chelators, moderate spiking is lost in the baseline noise. The final diet employed in our laboratories is a modification of the Wolfe diet;[13] we utilize ultrapure metal salts as a substitute for the commercial salt mix originally described in that diet. The final concentration of iron in this diet is 10

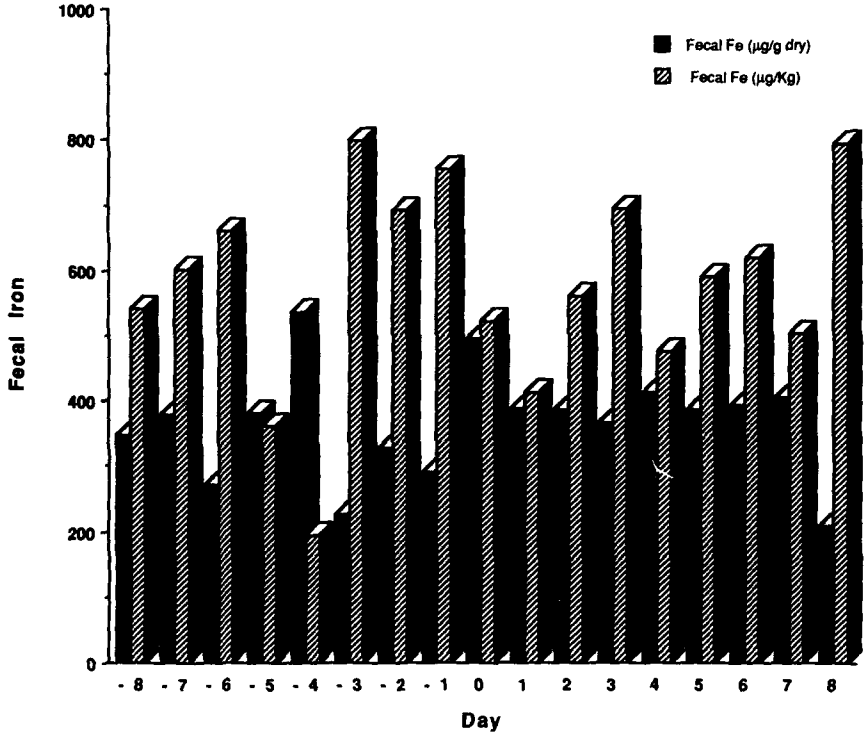


FIGURE 4. Fecal iron as determined in a monkey (8B6) maintained on Bio-Serv low-iron diet during treatment with desferrioxamine. The iron output is reported both as concentration in feces (µg/g dry weight) and as total output (µg/kg body weight). This diet led to rather substantial baseline noise.

ppm. The iron is concentrated to 75–100 ppm in the stool, and chelator-induced iron output is easily followed (FIGS. 5–6 and TABLES 2–3).

Anesthesia

Animals are anesthetized with Ketamine prior to drug administration and blood sampling. We observed that frequent administration of Ketamine can cause slight

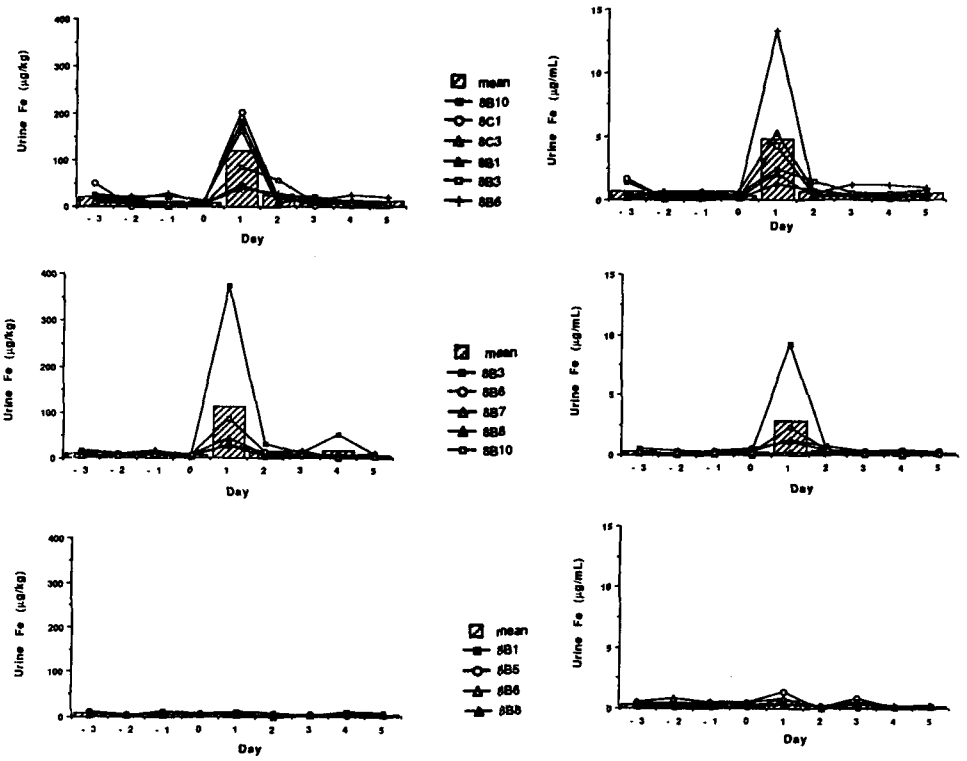


FIGURE 5. Drug-promoted urinary iron excretion in monkeys reported as total iron output in µg/kg (**left panels**) and as concentration in µg/ml (**right panels**). Drugs tested were desferrioxamine administered at 150 µmol/kg (**top panels**), desferrithiocin administered at 150 µmol/kg (**middle panels**), and PIH analogue administered at 300 µmol/kg (**bottom panels**).

increases in SGPT and SGOT levels. These values, however, returned to normal after several days. Because Ketamine decreases intestinal motility, it should not be given after the administration of the drug.

Chelator-induced Iron Clearance

The *Cebus* monkeys respond very differently to the ligands than do the rats. The PIH analogue is inactive, while desferrithiocin outperforms desferrioxamine. The efficiency of each ligand is calculated on the basis of a 1:1 desferrioxamine-iron complex, a 2:1 desferrithiocin-iron complex, and a 2:1 PIH-iron complex. The numbers are generated by averaging the iron output four days prior to administration of the drug, subtracting these numbers from the two-day iron clearance after administration of the drug, and dividing by the theoretical output. Our experiments indicate that chelator-induced iron clearance is over after two days.

Toxicity Evaluations

Behavioral, CBC and multiple serum analysis changes are used to evaluate the potential toxicity of each drug. Of the three ligands, only the PIH analogue produced any indications of a toxic response. This is reflected by vomiting, lethargy, and/or a refusal to eat and drink. These kinds of responses are not observed in rats, even at a dose ten times higher than that given the monkeys.

DISCUSSION

Monkey Model

Use of the monkey as a model for testing prospective iron chelators makes it possible to observe untoward reactions caused by the drug, reactions which are not seen in rodents. For example, with the PIH analogue we note vomiting, lethargy, and a decrease in food intake. We notice no such response in rodents. Furthermore,

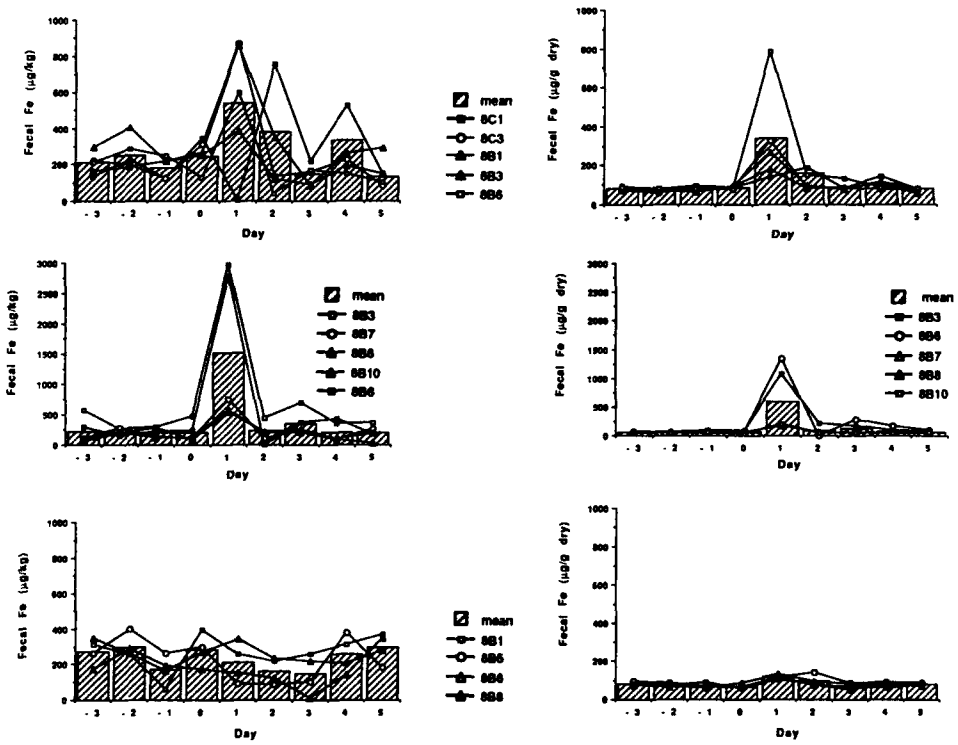


FIGURE 6. Drug-promoted fecal iron excretion in monkeys reported as total iron output in µg/kg (**left panels**) and as concentration in µg/g dry weight (**right panels**). Drugs tested were desferrioxamine administered at 150 µmol/kg (**top panels**), desferrithiocin administered at 150 µmol/kg (**middle panels**), and PIH analogue administered at 300 µmol/kg (**bottom panels**).

TABLE 2. Drug-induced Iron Excretion in the Urine and Feces of Iron-Overloaded *Cebus* Monkeys (*Cebus apella*)

	Iron Excretion[a]					
	DFO (150 μmol/kg, s.c.) $n = 6$		DFT (150 μmol/kg, p.o.) $n = 5$		PIH Analogue (300 μmol/kg, p.o.) $n = 4$	
Measurement	mg/kg	%[b]	mg/kg	%[b]	mg/kg	%[b]
Theoretical total	8.37	100	4.19	100	8.37	100
Experimental total	0.50 ± 0.22	5.9	1.40 ± 1.18	32.5	−0.14 ± 0.21	−1.70
In urine	0.12 ± 0.07	23.2	0.11 ± 0.15	7.9	0.00 ± 0.00	
In feces	0.39 ± 0.21	76.8	1.29 ± 1.08	92.1	−0.14 ± 0.21	

[a]Route of administration (s.c., subcutaneous; p.o., oral) and number of animals studied (n) are indicated for treatment with DFO (desferrioxamine B), DFT (desferrithiocin), and the PIH (pyridoxal isonicotinoyl hydrazone) analogue CGP43902B. The molecular weights and ligand: Fe binding ratios, respectively, for these drugs are (DFO) 656, 1:1; (DFT) 260, 2:1; and (PIH analogue) 442, 2:1.

[b]Values for urine and feces are calculated as % of corresponding total experimental value.

complete blood counts, blood chemistries, and liver function studies make it possible to observe any acute changes caused by the drug. As the baseline values are identical with those of humans, any changes are even more interesting for their possible relevance to human responses. The major difficulty with the monkey model is associated with the development of a proper diet. It must be appropriately designed to reduce the iron noise level in the stools to around 100 ppm, a situation which makes it possible to easily identify desferrioxamine-induced iron excretion in the feces. Desferrioxamine-induced iron clearance serves as a positive control for all of these studies. It can be seen from FIGURE 4 that if the diet is not properly adjusted, evidence of moderate fecal iron excretion can be lost in the noise. The diet has little if any effect on baseline urinary iron excretion.

Iron Loading

Iron dextran has been used clinically for the treatment of various iron-deficiency disorders, with a low incidence of undesirable effects.[15] Initially only intramuscular administration was employed, but later intravenous administration was found to be safe and more convenient for many patients. Intravenous iron dextran is removed slowly from the plasma and is taken up by the R.E. cells. The half-life of iron dextran in the plasma of humans is 2.5–3 days.[16] Although there are a number of ways to evaluate animal iron overload, we decided to deliver 500 mg/kg of the metal to each animal and to wait twenty half-lives, sixty days, to make seminal measurements. At

TABLE 3. Comparison of Chelator Efficiencies in Rats and Monkeys

	Iron Excretion (%)	
Compound	Rats	Monkeys
Desferrioxamine (DFO), s.c.	3.3	5.9
Desferrithiocin (DFT), p.o.	3.5	32.5
PIH analogue, p.o.	3.4	−1.7

this time there would be less than half a microgram per kilogram of iron in the blood associated with iron dextran. Liver biopsies in our animals show significant hemosiderin in both Kupffer cells and hepatocytes, with most of the iron in the R.E. cells (FIGS. 7–8). Transfusional iron overload causes a histologic picture similar to that caused by intramuscular or intravenous iron dextran loading.[17]

Iron dextran given intramuscularly is initially routed by way of the capillaries and

FIGURE 7. Liver biopsy stained with Prussian blue (iron stain). Monkey 8B1, weight 3.4 kg, loaded with 309 mg/kg iron. This monkey was given iron in two intravenous injections; the last injection was on November 30, 1988. The liver biopsy was done March 1990. Magnification: 40×.

lymph to the R.E. cells in a manner similar to that seen with intravenous infusions. We chose to give the iron dextran by the intravenous route as the method of iron loading the *Cebus* monkeys since it is a safe clinical procedure which enables us to administer the required iron in one or two infusions, thereby markedly decreasing the number of injections and anesthesia necessary for multiple intramuscular

injections. Since *Cebus* monkeys have limited musculature, the amount of intramuscular iron dextran given at one site at one time must be small.

Comparative Studies

At the very least, cell culture evaluations of iron chelators serve as excellent negative indicators of the potential activity of a ligand in animals. If an iron chelator

FIGURE 8. Liver biopsy stained with Prussian blue (iron stain). Monkey 8B6, weight 3.9 kg, loaded with 520 mg/kg iron. The iron loading was with five intravenous injections; the last injection was on June 23, 1988. The liver biopsy was done March 1990. Magnification: 40×.

does not remove iron from cultured cells, it is unlikely to remove iron from a whole animal. This is a critical issue, as there are thousands of compounds which qualify as potential therapeutic iron chelators based on their iron-binding properties. Surveying this number of ligands in a rodent model would be unrealistic in terms of time

and expense. Any assay system which can reduce these numbers is of great value. The rodent model has traditionally been the last step for chelator iron-clearance evaluation prior to human clinical testing. Although extensive toxicity studies are generally carried out on promising compounds after the rat model is used and before the clinical studies are conducted, questions as to the predictive value of the rat model for human studies still remain.

In an attempt to address this concern, we have compared the ability of three different iron chelators to clear iron in a non–iron-overloaded, bile-duct cannulated rat with their ability to clear iron in a *Cebus* monkey model. These ligands include desferrioxamine, desferrithiocin, and a pyridoxyl hydrazone derivative (CGP43902B). Desferrioxamine is administered subcutaneously as a positive control and orally as a negative control; desferrithiocin and the PIH analogue are given orally. Each drug is dispensed at 150 μmol/kg. PIH in the monkeys is delivered at 300 μmol/kg.

The most effective of the chelators in the rodent model is desferrioxamine given subcutaneously, with approximately 70% of the iron excreted in the bile and 30% in the urine. The urinary excretion is complete on day +1. Biliary clearance is essentially over in 9 h. The efficiency of the chelator averages 3.3%, calculated on the basis of a 1:1 complex. Desferrithiocin administered orally generates 88% of the iron in the bile and 12% in the urine, with an efficiency of 3.5%, based on the 2:1 complex. PIH generated 87% of the iron in the bile and 13% in the urine, with an efficiency of 3.4%, based on a 2:1 complex.

The response to desferrioxamine in the *Cebus* monkey model is somewhat different from that observed in the rat model. Although there is greater variability in the results in the monkeys (TABLE 2), 77% of the iron is excreted in the bile. The urinary excretion is complete on day +1, and the efficiency of the ligand is 5.9%. It is interesting that although the efficiency is higher in the monkey, most of the iron is cleared in the bile as with the rat. Desferrithiocin in the monkeys behaves somewhat differently than it does in the rat. Although 92% of the iron is cleared in the bile, the efficiency of the ligand is much higher in the monkeys, 32.5%. As with the desferrioxamine, the urinary clearance is complete on day +1. Finally, the response of the primates to the PIH analogue CGP43902B is remarkably different from that seen in the rat. There is little if any iron clearance in the bile or the urine. Furthermore, two of the four animals vomited the drug immediately after administration.

It is interesting that in both models the pattern of chelator-induced iron clearance is the same for desferrioxamine and desferrithiocin, i.e., most of the iron is excreted in the bile. However, not only is the efficiency of both chelators substantially higher in the monkeys, but desferrithiocin is far more active than is desferrioxamine in the monkeys. While the PIH analogue works well in the rodent model, it does not perform at all in the monkey model.

CONCLUSION

On the basis of the results of this comparative evaluation, we conclude that the modified secondary screen using iron-loaded *Cebus* monkeys will prove to be a more predictive test model for oral iron chelators than are the previously described rodent models. Major improvements of the analytic procedures and a special low-iron diet now allow an accurate determination of fecal iron excretion and thus of the overall iron output.

Of the three ligands performing about equally well in the rodent model, two were found to significantly promote iron clearance from the iron-loaded primates. The overall efficiency of desferrioxamine B was roughly comparable to its performance in

patients with iron overload. The iron clearance induced with desferrithiocin exceeded the level observed with any other chelator, indicating ready access to the main iron stores. Quite surprisingly, no increase of iron-clearance levels can be observed with the PIH analogue CGP43902. Moreover, various salt forms of this chelator caused vomiting and loss of appetite in some of the test animals.

This new primate screen will be used to complement the preclinical evaluation of other ligands which have shown promise as orally active iron chelators.

SUMMARY

A comparative study of the non–iron-overloaded, bile duct–cannulated rat and of the *Cebus* monkey as iron-clearance models is presented. The ability of desferrioxamine, desferrithiocin, and a pyridoxal isonicotinoyl hydrazone (PIH) analogue to clear the metal from these two animals is evaluated. Data suggest that although rodents represent a viable first-line animal screen, there is no strict correspondence between the effectiveness of a chelator in rodents and that in primates. Rodent data should be interpreted carefully as it relates to potential human trials. Iron-loading response, the similarity between multiple human and *Cebus* serum and hematological values, and the ability to easily observe changes in behavioral patterns clearly render the *Cebus* monkey the best preclinical screen.

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A New Approach to Bone Marrow Transplantation in Thalassemia

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The possibility of a cure for homozygous β-thalassemia by bone marrow transplantation was first demonstrated by Thomas *et al.* in a 16-month-old patient transplanted on December 2, 1981.[1] Our experience with this approach began on December 17, 1981, when a 14-year-old patient received a bone marrow transplant after preparation with cyclophosphamide and total body irradiation (TBI) without marrow engraftment. Of four consecutive patients successively transplanted, one died, two rejected the graft, and one—transplanted on July 22, 1982—is alive and cured. We therefore transplanted eight patients with various combinations of busulphan, cyclophosphamide, and TBI before conclusively adopting the regimen busulphan-cyclophosphamide, in January 1983.[2,3] In the present report we examine the results of marrow transplantation after preparation of the recipients with busulphan and cyclophosphamide in 350 patients with thalassemia, aged 1 through 21 years.

METHODS

Between January 1983 and February 1990, 350 patients between the ages of 1 and 21 years received HLA-identical marrow transplantation for the treatment of homozygous β-thalassemia. Of these patients, 336 received marrow from an HLA-identical sibling and 14 from an HLA-identical parent. All patients were prepared for transplantation with a modification of the regimen described by Santos *et al.*[4] Six patients received a total dose of busulphan of 16 mg/kg, and 344 received a total dose of 14 mg/kg. This treatment was followed by a total dose of cyclophosphamide of 200 mg/kg. Details on the protocol for bone marrow transplantation in thalassemia used by us in Pesaro are reported elsewhere.[5] Acute and chronic graft-versus-host disease was graded according to the Seattle criteria. Cytogenetic analysis and globin chain synthesis examined by column chromatography were used to analyze the graft. Liver biopsy was performed 15 days before transplantation. A grading system was established to assess siderosis, chronic aggressive or chronic persistent hepatitis, and portal fibrosis on the liver biopies. Three grades of severity (mild, moderate, and severe) were identified.[6] In evaluating the quality of chelation, establishment of a pattern of subcutaneous infusion of deferoxamine at 20–40 mg/kg for 8–12 h a day at least five days a week initiated not later than two years after red-cell transfusions began was considered to indicate good chelation. Failure to achieve this standard

TABLE 1. Bone Marrow Transplantation in 350 Patients with Thalassemia

Outcome[a]	Probability (%)
Survival	82
Event-free Survival	73
Rejection	12

[a]Minimum follow-up period was 2 months; maximum was 7 years.

was categorized as poor chelation. Survival and event-free survival were evaluated by the product-limit method of Kaplan and Meier[7] and tested for p value by the Mantel-Cox and Breslow statistic. Multivariate analysis was performed with the Cox proportional-hazard regression model.[8]

RESULTS

TABLE 1 reports the probabilities of survival, event-free survival, and rejection for all 350 patients transplanted. In 161 patients transplanted after preparation for the transplant with the regimen used for all patients from June 1985 through March 1989 (busulphan, 14 mg/kg; cyclophosphamide, 200 mg/kg; and cyclosporine alone; Protocol 6), the following factors were examined for their influence on survival and event-free survival: age, number of transfusions received before the transplant, ferritin level, quality of chelation, hepatomegaly, splenomegaly, splenectomy, hemosiderosis, chronic aggressive hepatitis, chronic persistent hepatitis, and portal fibrosis. The results of this analysis are summarized in TABLE 2. Three factors assessed at the time of transplantation were univariately associated with significantly reduced probability of survival and event-free survival: liver more than 2 cm below costal rib ($p = 0.0001$), poor quality of chelation treatment ($p = 0.002$), and presence of portal fibrosis ($p = 0.01$).

The 34 patients with none of these three significant factors were assigned to Class 1, while the 33 patients that presented with all the three factors were assigned to Class 3. The 100 patients with various combinations of only two of the three factors were assigned to Class 2. TABLE 3 shows the probabilities of survival and event-free survival for these three classes. The probabilities for patients in Class 1 were not significantly different from those in Class 2, but they were significantly different when

TABLE 2. Univariate Analysis of Risk Factors for Outcome of Transplant in 161 Patients Prepared for Bone Marrow Transplantation by Protocol 6[a]

Significant	Not Significant
Hepatomegaly ($p = 0.0001$)	Age
Portal fibrosis ($p = 0.01$)	Ferritin level
Chelation: regular vs. irregular ($p = 0.002$)	No. transfusions
	Splenomegaly
	Splenectomy
	Liver iron concentration
	Hemosiderosis
	Hepatitis

[a]Minimum follow-up period was 1 year.

TABLE 3. Bone Marrow Transplantation in 167 Patients with Thalassemia Transplanted after Preparation with Protocol 6: Division into Three Classes of Risk[a]

	Probability (%)			*p* Values		
Outcome[b]	Class 1 (n = 34)	Class 2 (n = 100)	Class 3 (n = 33)[c]	Class 1 vs. Class 2	Class 1 vs. Class 3	Class 2 vs. Class 3
Survival	97	86	58	0.06	0.0001	0.0004
Event-free survival	94	83	53	0.08	0.0001	0.0003
Rejection	3	6	12			

[a]An additional 16 patients, who did not have a suitable liver biopsy, have been excluded from categorization into classes.
[b]Minimum follow-up period was 1 year; maximum was 4.5 years.
[c]Input into Class 3 stopped in March 1989.

compared with those in Class 3. The probabilities for patients in Class 2 were also significantly different from those in Class 3.

For Class 3 patients, we modified the standard Protocol 6 in March 1989 as follows: busulphan, 14 mg/kg; cyclophosphamide, 120 mg/kg; anti-lymphocyte globulin (ALG), 10 mg/kg; cyclosporine and short-term methotrexate (Protocol 12). For patients in Class 1 and those in Class 2, the standard Protocol 6 continued to be used.

From March 1989 through March 1990, 14 patients were transplanted after preparation with Protocol 12. Actual survival and event-free survival are 100% and 79%, respectively. This study is still under evaluation, but the rate of early transplant-related deaths already appears to be reduced compared to that seen with Protocol 6 in Class 3 patients. TABLE 4 reports the results obtained with Protocol 12 in 14 Class 3 patients.

CONCLUSIONS

We conclude that patients with thalassemia who have an HLA-identical sibling or parent should be transplanted as soon as possible. If the results obtained with our Protocol 12 in Class 3 patients are confirmed, it will indicate that thalassemic patients of all ages with a suitable donor can have access to the possibility of a radical cure of their disease with bone marrow transplantation.

TABLE 4. Bone Marrow Transplantation in 14 Class 3 Thalassemic Patients Transplanted after Preparation with Protocol 12[a]

Outcome[b]	n	%
Survival	14	100
Alive with return of thalassemia	3	21
Event-free survival	11	79

[a]Input of patients to Protocol 12 was initiated in March 1989.
[b]Follow-up period was 2–12 months.

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Gene Transfer into Murine Hematopoietic Stem Cells and Bone Marrow Stromal Cells

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PRINCIPLES OF RETROVIRAL GENE TRANSFER

Retroviruses are an attractive vehicle for the transfer of new genetic material into cells. Upon infection of a permissive cell, the viral genome, consisting of single-stranded RNA, is copied by viral reverse transcriptase and cellular polymerase; and the resulting double-stranded DNA integrates into the host cell genome. With the aid of specialized packaging cell lines, it is possible to create recombinant replication-defective retroviruses, in which the majority of the viral sequences have been deleted and replaced with sequences that one desires to have integrated into the host cell genome.[3-8] The hematopoietic system is a particularly suitable target for this mode of gene therapy, because bone marrow can be harvested and manipulated extensively *in vitro* and only a small number of hematopoietic stem cells (HSC) are necessary for reconstitution of the hematopoietic system of an appropriately conditioned recipient. Candidate genes for retroviral-mediated gene transfer into the hematopoietic system have included β-globin,[9-12] adenosine deaminase (ADA),[1,13-16] purine nucleoside phosphorylase,[17] hypoxanthine phosphoribosyl transferase,[18] mutant dihydrofolate reductase (conferring methotrexate resistance),[19,20] and neomycin phosphotransferase (conferring G418 resistance).[21-24] Our own laboratory has focused primarily on ADA as a model for gene therapy. ADA deficiency is responsible for one form of the human disease severe combined immunodeficiency (SCID).

In order to achieve long-term expression of the transferred gene in the hematopoietic system, it is essential to achieve gene transfer into a pluripotent and reconstituting stem cell. Furthermore, it is probable that host cell division is required for integration of the retroviral genome within the target cell genome and, thus, for successful retroviral-mediated gene transfer. Therefore, many investigators have focused on achieving conditions for retrovirus infection of bone marrow cells in which the pluripotent stem cell is dividing. These studies are difficult, however, since

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there is no simple and rapid assay for the reconstituting HSC. The spleen colony-forming unit (CFU-S), while a more differentiated cell than the reconstituting HSC, is nevertheless a multipotent stem cell with limited self-renewal capacity.[25] Since the murine CFU-S can be assayed in clonogenic assays, it is a useful "approximation" of the pluripotent stem cell. Hodgson and Bradley demonstrated that 5-fluorouracil (5-FU) pretreatment of donor mice will increase the proportion of CFU-S stem cells in the harvested marrow by destroying more differentiated progenitor cells.[26] As HSC undergo division to replenish the depleted compartment of more differentiated progenitor cells, retroviral-mediated gene transfer is more efficient. 5-FU pretreatment of donor mice is now routine, and it is likely that it will be useful in larger animals as well. In a further effort to enhance retroviral gene transfer into hematopoietic stem cells, hematopoietic growth factors have been added to harvested bone marrow cells before and/or during the infection procedure. It is assumed that a proper combination of growth factors will support survival and stimulate self-renewing division of the HSC during the infection.

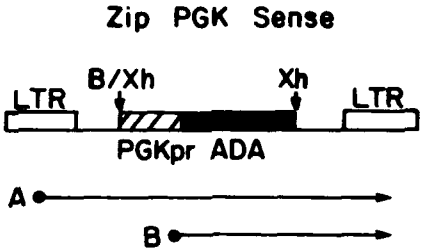

FIGURE 1. Zip PGK ADA retroviral construct (**upper panel**). (**Lower panel, A**) The viral genomic message; (**B**) the RNA transcript of the human ADA cDNA, driven off the PGK promoter (pr). Restriction sites: B, *Bam*H I; X, *Xho* I.

PRESTIMULATION OF BONE MARROW CELLS

Our own work has demonstrated the efficacy of WEHI-3b–conditioned medium (as a source of murine interleukin 3 [IL-3]) in improving the efficiency of retroviral infection of day 12 CFU-S ($CFU\text{-}S_{12}$)[1]. Bone marrow was infected with the simplified retrovirus, Zip PGK ADA, in which the cDNA for human ADA is expressed from the phosphoglycerate kinase (PGK) promoter (FIG. 1). Analysis of expression of transferred human ADA sequences is performed by a modification of the starch gel *in situ* enzyme assay. In these experiments, bone marrow was harvested from mice 48 h after 5-FU treatment. The marrow was then prestimulated in medium containing 10% WEHI-3b–conditioned medium (WEHI-CM) for various times and infected by cocultivation with the retroviral producer line in the continued presence of 10% WEHI-CM for various times. Subsequently, bone marrow cells were harvested and injected into lethally irradiated recipient mice. For the analysis of CFU-S–derived spleen colonies, $0.75–2.0 \times 10^5$ cells were injected into each recipient. Twelve days following transplant, animals were sacrificed and individual spleen colonies were dissected and examined for the presence of the proviral DNA and, by enzyme analysis, for the expression of the transferred human ADA sequences. The results, detailed in TABLE 1, show that a 48-h prestimulation period increased the proportion of infected CFU-S (expressing human ADA) from 18% without prestimulation to 39% with WEHI-CM prestimulation. Using this optimal 48-h prestimulation/48-h

cocultivation protocol, we studied mice for long-term expression of the transferred human ADA. Hematopoietic reconstitution of mice for these long-term expression studies was accomplished by injection of 3–6 × 10^6 infected bone marrow cells/mouse. One month post-transplant, 13/13 mice expressed human ADA in the peripheral blood. Four months post-transplant (after full hematopoietic reconstitution) 6/13 mice continued to express human ADA at easily detectable leveis in the peripheral blood. In more recent studies, using IL-1α, IL-6 (from B5637-conditioned medium) and IL-3 in combination, 75% of the mice at 6 months post-transplant demonstrate expression of human ADA in the peripheral blood (J. Apperley, B. Luskey and D. Williams; manuscript in preparation). It is noteworthy that achieving this high percentage of animals expressing the introduced ADA cDNA did not rely on any pretransplant selection for infected HSC. Bodine *et al.* have also reported the successful use of hematopoietic growth factors in similar infection protocols.[27]

TABLE 1. Effect of Prestimulation of Bone Marrow Cells on the Efficiency of Infection of CFU-S

Prestimulation[a] (h)	Cocultivation (h)	Human ADA–positive Spleen Colonies[b]
—	24	2/60 (3%)
48	24	24/159 (15%)
—	48	23/125 (18%)
48	36	21/62 (34%)
48	48	93/236 (39%)

[a]Prestimulation was in the presence of 10% WEHI-3b–conditioned medium.
[b]All spleen colonies examined which were positive for proviral insertion contained detectable human ADA. The results shown represent three repititions of the experiment.

THE ROLE OF THE HEMATOPOIETIC MICROENVIRONMENT

In vivo, hematopoietic stem cells are supported by the complex microenvironment of the bone marrow, which regulates HSC proliferation, differentiation, and self-renewal. No combination of known soluble growth factors can support the survival and proliferation of hematopoietic stem cells for long periods *in vitro.* However, long-term bone marrow cultures (LTMC; "Dexter cultures") provide an *in vitro* microenvironment made up of multiple cell types which can support stem cell proliferation and myeloid and lymphoid differentiation (Whitlock-Witte cultures).[28,29] Clearly, the stromal layers of Dexter cultures provide something necessary for the support of normal hematopoietic stem cell self-renewal, which requires direct stem cell–stromal cell contact.[30–32]

In an effort to develop a useful system to support hematopoietic stem cells for long periods of infection, manipulation, and/or drug selection *in vitro,* we have focused much of our work on the interaction of HSC with the hematopoietic microenvironment. We have used a defective recombinant retrovirus vector expressing a mutant of simian virus 40 (SV40) large T antigen to efficiently immortalize stromal cells from murine long-term marrow cultures.[2] As discussed below, several such immortalized cell lines generated by SV40 large T antigen expression support the maintenance of CFU-S_{12} and reconstituting hematopoietic stem cells *in vitro* for

up to four weeks. In these experiments, we used a retroviral construct consisting of the SV40 large T antigen sequences driven off the 5′ long terminal repeat (LTR). The gene for neomycin phosphotransferase (conferring G418 resistance) was downstream of the large T antigen sequences and was expressed from a spliced message initiated in the 5′ LTR. Following transfection of the Psi-2 producer cell line with this construct, a clone producing recombinant retrovirus (titer of 5×10^4 G418-resistant CFU/ml) was isolated. Supernatant from this producer line was used to infect the stromal layer of established murine long-term bone marrow cultures. Subsequently, G418-resistant clones were obtained, expanded into permanent cell lines, and examined.

These immortalized stromal cell lines were tested for their ability to support hematopoietic stem cells in long-term cultures. Following the establishment of a confluent stromal cell layer from cloned cell lines, culture flasks were charged with adherence-depleted bone marrow cells and cultured under standard long-term bone marrow culture conditions. Not only did hematopoietic islands develop in some of the cultures, but assay of the non-adherant cells for CFU-S_{12} demonstrated maintenance of CFU-S_{12} on some of the immortalized stromal cell lines for at least four weeks. In contrast, neither conditioned medium nor salt extracts from the same cell lines were capable of supporting CFU-S_{12} for more than one week *in vitro* in the absence of direct cell contact to the stromal cells. Moreover, one cell line, U2, supports the reconstituting stem cell for at least three weeks in culture. Bone marrow from a male donor, cultured on U2 for three weeks and transplanted into a female recipient, produced full hematopoietic reconstitution with male-derived bone marrow. One year post-transplant, Y chromosome–specific sequences were detected in DNA obtained from the thymus, spleen, and bone marrow of the female recipient (M. Rios and D. A. Williams, manuscript submitted).

Immortalized stromal cell lines may help provide useful insights into the microenvironment–stem cell interaction which is critical for the regulation of stem cell growth. Our laboratory is using such lines to study the adhesive interactions between stem cells and the microenvironment. In addition, these lines may be a useful source for the identification of novel hematopoietic growth factors. For example, we have recently identified and cloned a novel hematopoietic cytokine from a primate bone marrow–derived stromal cell line (S. Paul *et al.*, manuscript submitted).

Immortalized stromal cell lines may provide an *in vitro* hematopoietic microenvironment in which extensive infection and selection of hematopoietic stem cells without loss of their reconstituting ability is possible. To date, *in vitro* selection of infected cells prior to transplant has met with limited success in improving long-term expression of a retrovirally transduced gene. Vectors have been developed which contain not only the gene of interest but also a gene conferring on successfully transduced cells a selectable phenotype, such as G418 resistance (neomycin phosphotransferase, *Neo*^{r}), methotrexate resistance (mutant dihydrofolate reductase), or deoxycoformycin (dCF) resistance (ADA), thus allowing *in vitro* selection of infected cells prior to transplant. There is little doubt that, using vectors containing only *Neo*^{r}, selection with G418 for 48 h results in the efficient removal of non-transduced cells. Dick *et al.* first showed that the proportion of CFU-S containing the *Neo*^{r}-marked provirus approaches 100% after selection.[23] The use of G418 to select for cells capable of sustained expression of a second gene, however, has met with mixed success. Long-term expression of the transduced gene has not been improved. For instance, Karlsson *et al.* showed that mice transplanted with cells infected with a retrovirus containing both β-globin and *Neo*^{r} sequences and selected in G418 expressed human β-globin at four weeks, but they were all negative by 12 weeks post-transplant.[11] Marrow of these mice had been replaced by surviving host cells,

suggesting that pre-selection had completely eliminated long-term reconstituting stem cells. Thus, the HSC had not been efficiently infected during co-cultivation or the level of *Neo* expression was insufficient to ensure survival of HSC in G418.

One approach to this problem is to provide the appropriate microenvironment *in vitro* which would permit drug selection of infected bone marrow while preserving reconstituting hematopoietic stem cells. This is especially critical with gene transfer of ADA using simplified (i.e., "one gene") vectors, since successful selection of various cell lines with dCF for ADA expression requires at least 7–10 days' exposure to the drug (unpublished observations), and reconstituting hematopoietic stem cells survive only a short time *in vitro* without the support of a hematopoietic microenvironment. Therefore, we have generated a stromal cell line resistant to dCF by introduction and over-expression of the ADA gene in the U2 stromal cell line. Use of this dCF-resistant U2 stromal cell line (U2-dCF^r) should allow maintenance of hematopoietic stem cells into which human ADA has been introduced during this long *in vitro* selection process. The general approach is illustrated in FIGURE 2.

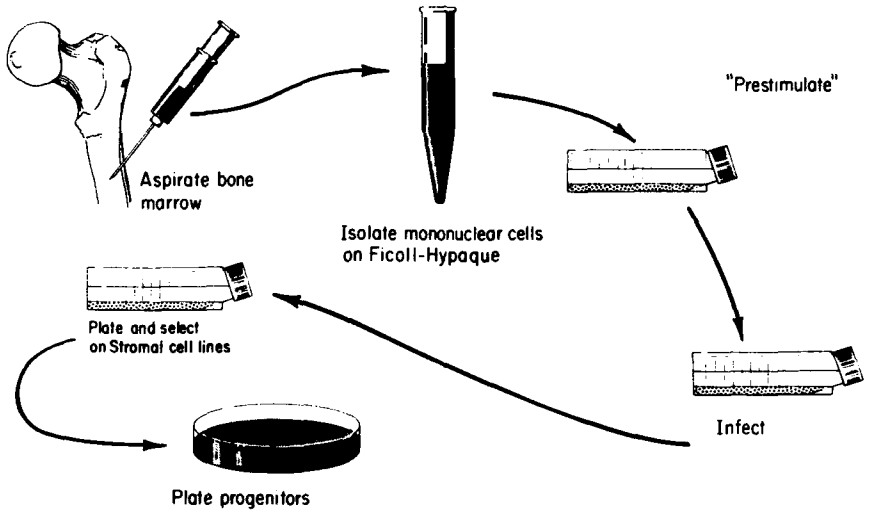

FIGURE 2. Proposed scheme for retroviral infection and selection of bone marrow cells. Primate or human bone marrow may be analyzed for progenitors able to grow on methylcellulose as shown. Murine bone marrow may be analyzed for CFU-S.

In preliminary experiments, as a model for future work, we have examined the ability of U2-dCF^r to maintain a multipotent, factor-dependent cell line (FDCP-Mix cells) during prolonged selection in dCF. FDCP-Mix is an IL-3–dependent hematopoietic cell line derived from murine long-term bone marrow cultures.[33] Although dependent on IL-3 for growth in liquid cultures, FDCP-Mix cells proliferate in long-term marrow cultures without added IL-3. Similarly, U2 cells support the growth of FDCP-Mix cells without added IL-3 and without loss of the FDCP-Mix multipotent phenotype (unpublished observations). FDCP-Mix cells were infected by cocultivation with an amphotropic producer of PGK-ADA in the presence of IL-3. Following infection, infected cells were cultured without added IL-3 on the U2-dCF^r with or without dCF (4 n*M*) drug selection. After five days the cultures were

completely depleted of non-adherant cells; the residual cells were then cultured in continued conditions of drug selection for an additional 5 days. Less than 1% of control (uninfected) cells survived the drug selection over this 10-day period. In contrast, a large population of dCF-resistant infected FDCP-Mix cells emerged during this same selection period. When assayed for clonogenic growth in semi-solid medium (methylcellulose) with and without dCF, only 10% of the infected unselected FDCP-Mix cells were resistant to dCF. In contrast, over 90% of the selected cells were resistant to dCF in the semi-solid medium. We intepret these results to show that proliferation of transduced FDCP-Mix clones occurred in association with drug-resistant stromal cells. Such proliferation occurred while FDCP-Mix cells maintained primitive characteristics such as clonogenic capacity. This system is a model for the infection and selection of primary hematopoietic stem cells on immortalized stromal cell lines.

Another approach to selection of transduced hematopoietic stem cells would be the use of a selectable gene for which *in vivo* treatment is possible. Our group has shown that the use of several retrovirus vectors which transfer a mutant dihydrofolate reductase cDNA (conferring methotrexate [MTX] resistance) into murine bone marrow cells can protect mice during the post-transplant period from lethal methotrexate-induced bone marrow aplasia.[19] However, with intermittent MTX administration, no obvious selection for transduced hematopoietic stem cells was noted.[20] It is possible that constant drug infusion would prove more valuable in effecting *in vivo* selection, since the half-life of MTX is very short *in vivo*. This approach is limited also by the number of drugs which exhibit bone marrow aplasia as the dose-limiting toxicity.

Considerable progress has been made in achieving gene transfer into the hematopoietic system using recombinant retroviral vectors. Clearly, it is possible to infect the reconstituting hematopoietic stem cell of the mouse and see long-term expression of the transduced gene in the myeloid and lymphoid progeny of this stem cell. Nevertheless, obstacles remain. The efficiency of retroviral-mediated gene transfer into the hematopoietic stem cell must be improved before this can be considered a feasible treatment modality. In this paper we have discussed our successful experience in using WEHI-CM to facilitate gene transfer into CFU-S and into hematopoietic stem cells. The use of purified or partially purified stem cells may improve the efficiency of infection; however, one must find conditions which promote cycling of these stem cells (thus rendering them susceptible to retroviral infection/integration) without loss of their reconstituting potential.[34-36] Investigators have not yet been completely successful in using drug selection protocols to achieve 100% efficiency of gene transfer into hematopoietic stem cells. We believe that at least part of the difficulty lies in the loss of reconstituting ability of hematopoietic stem cells when they are manipulated extensively *in vitro*. In this paper, we have discussed our successful experience in obtaining immortalized bone marrow stromal cell lines that have the capability of supporting hematopoietic stem cells for long periods in culture. In addition to aiding substantially in the study of the hematopoietic microenvironment, these stromal cell lines should prove useful in maintaining the viability of retrovirally transduced stem cells during a drug selection procedure.

SUMMARY

The use of recombinant retroviral vectors to transfer genetic sequences into hematopoietic stem cells (HSC) is one approach to somatic gene therapy. Two limitations of such retroviral vectors are the degree of efficiency of transfer into the

reconstituting hematopoietic stem cells and the loss of reconstituting ability of hematopoietic stem cells when manipulated *in vitro* during infection and selection. We have investigated the effects on the efficiency of gene transfer of prestimulation of hematopoietic stem cells by growth factors prior to infection. Prestimulation of bone marrow cells in WEHI-3b–conditioned media improved the efficiency of gene transfer into CFU-S stem cells.[1] The majority of animals transplanted with bone marrow infected after prestimulation with a simplified retrovirus, Zip PGK ADA, demonstrated long-term and stable expression of human adenosine deaminase (ADA) after full hematopoietic reconstitution. In separate experiments, retroviral vectors have been used to transfer the SV40 large T antigen sequences into stromal cells making up the hematopoietic microenvironment.[2] Stromal cells expressing large T antigen are immortalized, and some support the maintenance of day 12 CFU-S ($CFU\text{-}S_{12}$) and reconstituting hematopoietic stem cells *in vitro* for up to 4 weeks. Such immortalized stromal cell lines provide an *in vitro* hematopoietic microenvironment which may allow prolonged *in vitro* manipulations during infection and selection of hematopoietic stem cells without loss of reconstituting ability. We are using immortalized stromal cell lines resistant to deoxycoformycin (dCF) to select transduced murine HSC containing human ADA *in vitro*. The use of recombinant retroviral vectors provides a promising approach to correction of human diseases involving bone marrow cells.

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Retroviral Gene Transfer Using Safe and Efficient Packaging Cell Lines

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INTRODUCTION

The introduction of normal genes into the dividing cells of individuals affected by genetic disorders, termed gene therapy, has been the focus of much research over the past decade. Retroviruses appear to be the method of choice as vehicles to deliver exogenous genes into human cells. This is due to the high efficiency of retroviral gene transfer, as well as the fact that the retroviral proteins necessary to form the virion particle can be supplied in *trans*. The retroviral gene transfer system employs the use of two types of virus: a retroviral vector containing the gene to be transferred, and the helper or "packaging" virus which provides *gag, pol* and *env* proteins in *trans*. The vector virus contains the viral long terminal repeats (LTRs) and the packaging sequence but lacks the *gag, pol* and *env* gene sequences. The helper virus, contained in a packaging cell line (such as $\psi 2^1$), has a deletion of the ψ packaging sequence, which is required in *cis* for the packaging of retroviral RNA into the virion.

An important prerequisite for the use of retroviruses for gene therapy is the availability of safe retrovirus packaging cell lines incapable of producing wild-type virus.[2] The major danger of the use of retroviral vectors for gene transfer is the possibility that replication-competent viruses could be generated through recombination events in which the intact ψ sequence from the vector virus corrects the deleted ψ sequence of the helper virus. The proliferation of wild-type virus can lead to multiple integrations into the genome, which may result in the activation of potentially harmful genes such as oncogenes.[3,4] Packaging cell lines containing additional mutations, including deletions of the 3′ LTR and portions of the 5′ LTR in the PA317 cell line,[5] have been constructed as safer alternatives to the $\psi 2$ cell line. When PA317 cells are used, two recombination events are necessary to form a wild-type genome. Nevertheless, results from several laboratories indicate that, even when several mutations are present, wild-type virus can still be generated using PA317 cells.[6,7]

We have approached the problem of creating safe and efficient packaging cell lines by separating the viral *gag, pol,* and *env* genes of the helper virus onto two

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plasmids; *gag* and *pol* are on one plasmid and *env* is on the other. In addition, the ψ packaging sequence and the 3′ LTR have been removed in both plasmids. When the resulting ecotropic (GP+E-86) and amphotropic (GP+*env*Am12) packaging lines are used, at least three recombination events between the helper virus genome and the vector virus genome are necessary to generate a wild-type virus. Generation of replication-competent virus has not been detected with these packaging cell lines, and their efficiency of gene transfer is comparable to that of packaging lines containing the viral genes on one plasmid. These cell lines have also been demonstrated to be successful for use in gene transfer into live mice.

RESULTS

Generation of the Ecotropic Packaging Line

To generate the ecotropic packaging cell line, two helper virus plasmids, p*gag-pol*gpt[8] and p*env*[8] were constructed using Moloney murine leukemia virus (Mo-MULV) proviral DNA from the plasmid 3PO[9] (FIG. 1). The plasmid p*gag-pol*gpt was

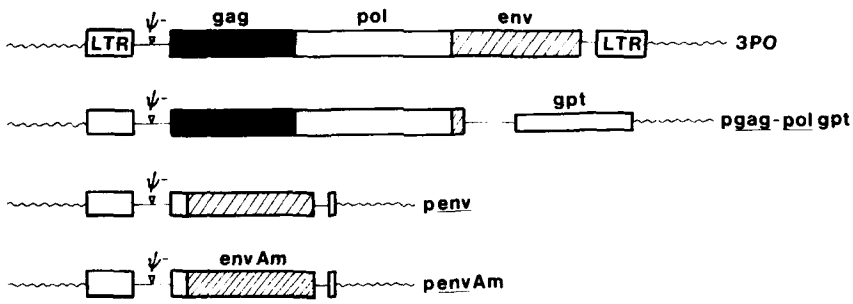

FIGURE 1. Comparison of viral sequences contained in plasmid 3PO and constructs p*gag-pol*gpt, p*env*, and p*env*Am. Mo-MULV LTRs and the ψ deletion (ψ⁻) are indicated. *Solid boxes* represent *gag* sequences; *open boxes, pol* sequences; *hatched boxes, env* sequences; *wavy lines,* pBR322 sequences.

constructed by isolating a fragment containing the 5′ LTR and the *gag* and *pol* DNA from 3PO and inserting this fragment into the plasmid pSV2gpt.[10] The plasmid p*env* was constructed by isolating a fragment from 3PO that contains the 3′ acceptor splice site and the *env* gene and ligating it to another fragment from 3PO containing the 5′ LTR and 5′ donor splice site.

3T3 cells were transfected by electroporation with p*gag-pol*gpt and p*env* DNAs, and recipient cells were selected for the presence of the *gpt* gene with media containing mycophenolic acid (MA).[8] MA-resistant (GP+E) clones were isolated, and their supernatants were tested for reverse transcriptase (RT) activity.[8,11] GP+E clones which produced high levels of RT were then tested for *env* protein production by immunoprecipitating labeled cellular proteins with anti-*env* antisera.[8]

Five GP+E cell lines which expressed high levels of RT and *env* proteins were tested for their ability to package the Δneo retroviral vector[8] (FIG. 2) These cell lines were transfected with Δneo DNA. G418-resistant clones were isolated and tested for their release of *neo* gene–containing viral particles by using harvested supernatants

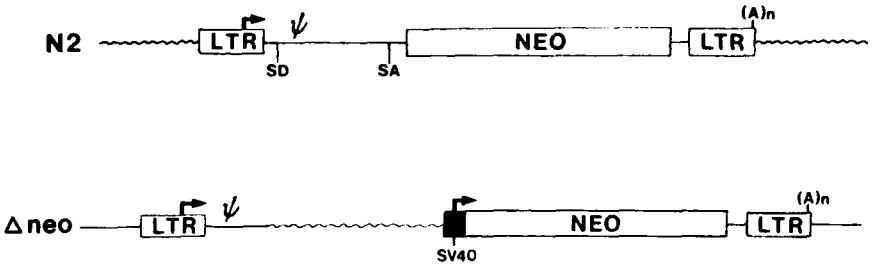

FIGURE 2. Replication-defective retroviral vectors N2 and Δneo. ψ, packaging sequence; *wavy line*, pBR322 sequences; *solid box*, SV40 promoter and origin of replication. SD, 5′ splice donor site; SA, 3′ splice acceptor site.

to infect 3T3 cells.[8] The GP+E-86 packaging cell line produced titers of Δneo (2.0×10^2 to 6.5×10^5 colony-forming units [CFU]/ml) that were consistently higher than those produced by any of the other four GP+E lines (TABLE 1 and additional data not shown). Titers were comparable to those obtained from the 3PO-18 packaging line,[8] which was generated by co-transfecting the plasmids 3PO and pSV2gpt into 3T3 cells.

TABLE 1. Virus Production From Ecotropic Packaging Cell Lines

Packaging Line	Vector	Clone[a]	Titer (CFU/ml)[b]
GP+E-86	Δneo	1	1.2×10^5
		8	3.0×10^4
		11	9.0×10^4
		17	6.5×10^4
		21	1.7×10^5
3PO-18	Δneo	1	2.2×10^4
		2	1.1×10^4
		3	5.7×10^3
		4	8.0×10^2
		5	6.7×10^3
GP+E-86	N2	3	1.2×10^6
		7	3.5×10^6
		8	2.6×10^6
		9	3.0×10^6
		11	1.3×10^6
		12	4.0×10^6
		13	3.6×10^6
3PO-18	N2	1	3.8×10^4
		2	1.8×10^4
		3	4.0×10^4
		6	1.0×10^5
		9	6.0×10^4
		10	1.0×10^5
		11	1.7×10^5

[a]Representative clones are shown.
[b]CFU, colony-forming units.

GP+E-86 cells were also tested for their ability to package the N2 retroviral vector[12] (FIG. 2). N2 DNA was transfected into GP+E-86 cells, and G418-resistant clones were isolated. Supernatants were then used to infect 3T3 cells. Titers of N2 virus ranged from 5.3×10^3 to 4.0×10^6 CFU/ml (TABLE 1). These titers were also comparable to those obtained using the 3PO-18 packaging line.

Generation of the Amphotropic Packaging Line

In order to create a packaging line to transfer retroviral vectors into human and primate cells, we substituted a plasmid containing an amphotropic *env* gene, p*env*Am,[13] for the ecotropic *env* gene in p*env*. The plasmid p*env*Am was constructed using DNA from the plasmid pL1,[14] which contains the 4070A amphotropic murine leukemia virus proviral DNA.[15] A fragment containing the *env* gene and the 3′ acceptor splice site was isolated and ligated to a fragment from 3PO containing the Mo-MULV 5′ LTR and 5′ donor splice site. The amphotropic packaging line was generated by first transfecting the p*gag-pol*gpt plasmid into 3T3 cells and selecting a MA-resistant clone, GP101, that produced a high level of RT.[13] The plasmids p*env*Am and pRSVhyg[16] were then co-transfected into GP101 cells. Clones resistant to 200 μg/ml hygromycin B[17] were isolated and tested for amphotropic *env* protein production by metabolic labeling followed by immunoprecipitation with anti-*env* antiserum.[13] The clone GP+*env*Am12 was selected for use because it was the cell line which produced a significantly higher level of amphotropic *env* protein than the other clones tested.

To test for packaging ability, GP+*env*Am12 cells were transfected with the N2 retroviral vector. G418-resistant clones were isolated, and titers of released N2 virus were determined by infecting 3T3 cells with harvested supernatants. The N2 viral titer produced by the GP+*env*Am12+N2 clones ranged from $< 10^2$ to $> 10^6$ CFU/ml (TABLE 2). In a control experiment, N2 was transfected into the PA317 amphotropic packaging cell line. Titers of G418-resistant clones, when used to infect 3T3 cells, ranged from $< 10^2$ to 3.3×10^5 CFU/ml. A number of N2-transfected GP+*env*Am12 clones were also tested for their amphotropic packaging ability by using super-

TABLE 2. N2 Virus Production From Amphotropic Packaging Cell Lines

Packaging Line	Clone	Titer (CFU/ml)[a]		
		3T3	K562	HeLa
GP+*env*Am12	3	2.6×10^5	6.0×10^3	3.0×10^4
	4	2.0×10^5	4.0×10^3	1.3×10^4
	6	1.7×10^5	1.0×10^3	3.5×10^4
	11	1.0×10^6	5.3×10^3	2.7×10^5
	12	1.0×10^5	1.0×10^2	5.4×10^4
	16	3.0×10^5	5.0×10^3	5.1×10^4
	Pool[b]	1.0×10^6	6.4×10^4	5.6×10^3
PA317	4	5.3×10^4	1.0×10^3	1.0×10^3
	5	3.6×10^4	1.4×10^4	1.0×10^3
	6	1.0×10^4	6.0×10^2	3.0×10^2
	9	2.0×10^4	1.0×10^4	1.7×10^3
	10	3.3×10^5	3.6×10^3	2.5×10^3
	Pool[b]	8.0×10^3	$< 10^2$	8.0×10^2

[a]Titers were determined on three different cell types, as indicated.
[b]Pools represent > 100 clones.

TABLE 3. Virus Production from GP+E-86 Cells Infected with N2 (Transfect-Infect Method)

Clone	Titer (CFU/ml)
1	3.3×10^6
2	7.9×10^6
3	3.2×10^6
4	7.2×10^6
5	1.4×10^7
6	1.0×10^7
7	1.4×10^7
8	4.2×10^6
9	6.6×10^6
10	9.1×10^6

natants to infect human K562[18] and HeLa[19] cells. Titers on K562 cells ranged from 1.0×10^2 to 6.4×10^4 CFU/ml. Titers on HeLa cells ranged from 5.6×10^3 to 2.7×10^5 CFU/ml. These titers are comparable to those obtained with supernatants from PA317 cells.

High-titer retroviral producer cells were also generated by the use of both the GP+E-86 ecotropic packaging cells and the GP+*env*Am12 packaging cells. The N2 retroviral vector was transfected into GP+*env*Am12 cells by electroporation, and supernatant from pooled clones was used to infect GP+E-86 cells. The resulting "transfect-infect" clones produced titers as high as 1.4×10^7 CFU/ml when assayed on NIH 3T3 cells (TABLE 3).

Analysis for Recombinant Infectious Retrovirus

A provirus mobilization assay was utilized as a stringent test for replication-competent retrovirus which may have been generated through three recombination events between the helper genomes in the GP+E-86 and GP+*env*Am12 cell lines and the N2 vector. The provirus mobilization assay was designed to detect a transfer of replication-competent helper virus or packaging function. Supernatants from the packaging lines and from packaging lines producing N2 virus were used to infect 3T3 cells harboring the N2 provirus (3T3:N2 cells) and 3T3 cells harboring a *his* provirus, which allows histidine-independent cell growth (3T3:116 cells). If the supernatants used in these infections contained replication-competent helper virus, the infected 3T3:N2 cells or 3T3:116 cells would begin to secrete N2 or *his* virus particles. The infected cells were tested for N2 or *his* virus production by using harvested supernatants to infect fresh 3T3 cells; this was followed by selection with 800 μg/ml G418 (for cells infected with N2) or with 1 mg/ml L-histidinol (for cells infected with *his*). Results from provirus mobilization assays indicated that neither the GP+E-86 and GP+*env*Am12 packaging lines nor these packaging lines containing the N2 retroviral vector produce replication-competent helper virus (TABLE 4). Provirus mobilization occurred only when supernatants from 3T3 cells producing wild-type retrovirus (3T3:NCA cells) were used to infect 3T3:N2 cells or 3T3:116 cells.

In another type of long-term safety analysis, GP+E-86 cells and GP+*env*Am12 cells were transfected with N2, and pools containing 500–1000 clones were collected. Supernatants from the GP+E-86 pools were used to infect 3T3 cells, and supernatants from the GP+*env*Am12 pools were used to infect 3T3, K562 and HeLa cells.

TABLE 4. Provirus Mobilization Assay for Helper Virus Production

Cells Tested	N2 Provirus Mobilization	*his* Provirus Mobilization
GP+E-86	–	–
GP+E-86 + N2 clone 12	–	–
GP+E-86 + N2 pools	–	–
GP+*env*Am12	–	–
GP+*env*Am12 + N2 clone 11	–	–
GP+*env*Am12 + N2 pools	–	–
3T3	–	–
HeLa	–	–
K562	–	–
3T3:NCA	+	+

The infected cells were passaged for one month without G418 selection, to allow for the possible spread of a rare recombinant wild-type virus throughout the cell population. Supernatants from these infected cells (secondary supernatants) were then harvested and analyzed for wild-type virus by testing for RT activity and also by infecting 3T3 and HeLa cells and testing for G418-resistant cells. All of these supernatants were RT-negative and did not confer G418 resistance upon reinfection of 3T3 or HeLa cells (TABLE 5).

Transfer of the Neo^R *Gene into Irradiated Mice*

The *neo*^{R} (neomycin-resistance) gene of the N2 retroviral vector was transferred into irradiated mice to determine if the GP+E-86 and GP+*env*Am12 packaging lines are capable of transferring an exogenous gene into hematopoietic cells. Retroviral gene transfer and bone marrow transplantation were performed as described by Hesdorffer *et al.*[20] Marrow was harvested from the hind limbs of donor C57BL/6J mice which had received 48 h of treatment with 5-fluorouracil (500 mg/kg). The bone marrow was infected with N2 virus by coculturing with GP+E-86 high-titer producer cells (4×10^6–1.4×10^7 CFU/ml) for 48 h and then was selected in 2 mg/ml G418 for 24 h. After selection in G418, 1×10^6 nucleated cells were injected into the tail veins of irradiated recipient mice.

Transplanted mice were sacrificed at various time points, ranging from 12 days to 310 days post-transplantation. DNA was extracted from spleen colonies (from 12-day post-transplant mice), whole spleens, and bone marrow.[20] DNA samples were

TABLE 5. Long-Term Assay for Detection of Wild-Type Retrovirus

Cells Tested	Cells Infected	Cells Infected with Secondary Supernatant	G418-resistant Cells	RT
GP+*env*Am12 + N2 clone 11	3T3	3T3	–	–
GP+*env*Am12 + N2 pools	3T3	3T3, HeLa	–	–
GP+*env*Am12 + N2 pools	HeLa	3T3, HeLa	–	–
GP+*env*Am12 + N2 pools	K562	3T3, HeLa	–	–
PA317 + N2 pools	3T3	3T3, HeLa	–	–
GP+E-86 + N2 pools	3T3	3T3, HeLa	–	–

digested with appropriate restriction endonucleases and analyzed by Southern blotting of agarose gels using a 1.5-kilobase (kb) *neo*^{R} fragment as a probe.

The results of Southern blot analysis of spleen and marrow DNA digested with *Eco*R I (which cuts the N2 provirus at either end of the *neo*^{R} gene) indicate the presence of the *neo*^{R} gene between day 12 and day 200 following transplantation. The efficiency of gene transfer into irradiated mice ranged from 48% to 52% in the transplanted mice (11/17 mice in one experiment and 6/11 mice in another experiment). Whole spleen DNAs from these mice were also digested with *Hin*d III, which does not cut within the N2 provirus, to determine the number of viral integration sites per transplanted mouse. The number of viral integration sites reflects the number of infected hematopoietic stem cells that repopulated the transplanted mouse, assuming one viral integration per stem cell. Between three and six integration sites were apparent in the spleen DNAs from transplanted mice.

SUMMARY

One of the requirements for the use of retroviral vectors in human gene therapy is a packaging cell line which is incapable of producing replication-competent virus and which produces high titers of replication-deficient vector virus. Wild-type virus may be produced through recombinational events between the helper virus and a retroviral vector. We have constructed an ecotropic packaging cell line, GP+E-86, and an amphotropic packaging cell line, GP+*env*Am12, in which the viral *gag* and *pol* genes are on one plasmid and the viral *env* gene is on another plasmid. Both plasmids contain deletions of the packaging sequence and the 3′ LTR. The fragmented helper virus genomes, when introduced into 3T3 cells, produce titers of retrovirus which are comparable to the titers produced from packaging cells containing the helper virus genome on a single plasmid. We have found no evidence for the generation of wild-type retrovirus using the GP+E-86 and GP+*env*Am12 packaging lines, either alone or in combination with the N2 retroviral vector. We also show that these packaging cell lines can be used to transfer the *neo*^{R} gene of the N2 vector into mouse hematopoietic cells, followed by successful (48–52%), long-term (up to 200 days) transplantation into irradiated recipients. These results indicate that these packaging lines are safe and efficient for use in experiments designed for murine (using GP+E-86) and human (using GP+*env*Am12) gene therapy.

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Development of a High-Titer Retrovirus Producer Cell Line and Strategies for Retrovirus-mediated Gene Transfer into Rhesus Monkey Hematopoietic Stem Cells

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INTRODUCTION

All cells in the peripheral circulation are descendents of a pluripotent hematopoietic stem cell (PHSC). This cell has the capacity to self-renew or become committed to the myeloid or lymphoid lineages (for review see Ref. 1). The ability to introduce exogenous genetic material into these cells may provide a new type of therapy for certain heritable disorders affecting the hematopoietic system.[2,3] Retroviral-mediated gene transfer has been used to transfer several genes into murine bone marrow cells, including the genes for human β-globin,[4-6] human adenosine deaminase (ADA),[7-9] murine interleukin-3,[10] murine granulocyte-macrophage colony-stimulating factor,[11] murine dihydrofolate reductase,[12] and bacterial neomycin resistance (*neo*^{r}).[13-16]

The efficiency of retroviral-mediated gene transfer into murine stem cells is influenced by the titer of the recombinant virus,[7,9] preconditioning of the donor marrow with 5-fluorouracil (5-FU),[13,14,17] the timing and duration of exposure to virus,[5,8] and the presence of hematopoietic growth factors during culture of bone marrow cells *in vitro*.[5] Titer is defined as the concentration of infectious retroviral particles that accumulates in the culture medium of producer cells during 24 h of growth by the cell culture from a subconfluent to a confluent state. Most producer clones useful for gene transfer into murine stem cells have titers of 10^6–10^7 infectious retroviral particles per milliliter. Lim *et al.*[8] and we[5] have shown that gene transfer to murine PHSC is enhanced by extending the infection period, presumably by allowing time for stem cells to enter the cell cycle. We have reported that interleukin-3 (IL-3) and interleukin-6 (IL-6) synergize to increase both the CFU-S (spleen colony-forming unit) number and competitive repopulating ability of cultured stem cells[5] and to improve the frequency of gene transfer to murine PHSC.

To attempt gene transfer in a large animal model, we have developed an amphotropic retrovirus producer cell line that secretes gibbon IL-3 and human IL-6.

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Using a superinfection strategy, we have increased retrovirus production by this cell line to greater than 10^{10} functional viral particles per milliliter of culture medium. This cell line has been used to reproducibly transfer genes into bone marrow stem cells of rhesus monkeys.

METHODS

Cells and Viruses

The ψβS1 producer line has a titer of 2×10^6 *neo*^{r} CFU/ml.[17] All pA317[18] cells and their derivatives were maintained in Improved Minimal Essential Medium (IMEM; Biofluids) with 10% fetal calf serum (FCS; ABI). GP+E-86 cells[19] were maintained in Dulbecco's modified Eagle's medium (DMEM; Biofluids) with 10% newborn calf serum (NCS; GIBCO).

Growth Factors

The biological activities of murine IL-3, gibbon IL-3, and human and murine IL-6 were assayed on 32D, MO7E, and T1165 cells respectively, using known standards as controls.

Mice

WBB6F1-*W/W*^{v} (recipients for long-term gene transfer experiments) and female C57BL/6J (donors for all experiments) were purchased from the Jackson Laboratory. Young (4–6 weeks old) donor mice were treated with 150 mg/kg 5-FU (Fluka) intravenously 48 h before bone marrow harvest.

Amplification of Retrovirus Production

A pA317 cell line producing 5×10^6 infectious particles/ml of the N2[20] virus was generated by standard techniques.[21] This cell line was cotransfected with the plasmids pXM-gIL-3,[22] pXM-IL-6,[23] and pMohgr, which contain the gibbon IL-3, human IL-6, and the hygromycin resistance genes, respectively. A population of transfected cells (designated N236) was isolated by selection in 200 μg/ml hygromycin (Sigma). N236 and GP+E-86 cells were mixed in ratios of 3:1, 1:1, and 1:3. A total of 5×10^5 cells from these mixtures was seeded onto 10-cm plates in 10 ml of DMEM with 10% NCS. These cultures were split 1:3 upon reaching confluence, usually every 2–3 days, for a period of 17 days. On day 17 of co-culture, the supernatant of each culture was assayed for virus production; the cultures were plated out at limiting dilutions in IMEM with 10% FCS and 200 μg/ml hygromycin, and individual amphotropic clones were isolated.

Primate Gene Transfer Protocol

On day 1, confluent plates of producer cells were split 1:10 in DMEM, 10% FCS (Hyclone), and 6 μg/ml Polybrene (Sigma). Bone marrow cells were aspirated from

adolescent (ca.4-kg) rhesus monkeys in DMEM plus 2% FCS (Hyclone) and 10 U/ml heparin. Mononuclear cells were isolated by centrifugation through LSM-Lymphocyte Separation Medium (Organon Teknika) as directed by the supplier. Approximately 1–2 × 10^7 cells were added to each plate, and co-cultivation was allowed to proceed for 3 days. On day 3 the non-adherent cells were collected from the original plates and reseeded for an additional 3 days on fresh plates prepared as on day 1. On days 3 and 4, the donor animal was given 500 rads of total body irradiation. On day 6, non-adherent cells were collected, washed in Hanks' balanced salt solution, and infused into the irradiated donor in 50 ml of phosphate buffered saline plus 2% FCS (Hyclone) and 10 U/ml heparin. In some experiments, human IL-3 (ca.200 U/ml) was added to the cultures. Forty-eight hours after autologous transplantation, a continuous infusion of GM-CSF (50 U/kg/day) was administered to shorten the period of neutropenia following irradiation.[24] The murine gene transfer experiments used a protocol similar to the protocol described above except that 48 h after the 5-FU treatment, unfractionated bone marrow from C57BL/6J donor mice was used.

Retrovirus Titer Assays

Retrovirus production (titer) by amphotropic producer cell lines was assayed on HeLa cells, while the titer of mixed pools of producer cells or ecotropic producer cells was assayed on NIH 3T3 cells. Plates (6 cm) were each seeded with 1 × 10^5 HeLa or NIH 3T3 cells in 5 ml IMEM plus 10% FCS. After 24 h, the medium on these plates was replaced with 5 ml of serial 10-fold dilutions of medium conditioned for 24 h by confluent cultures of producer cells. Polybrene was added to a final concentration of 6 μg/ml. Twenty-four hours later, the virus-containing medium was replaced with fresh medium. After another 24 h, the medium was replaced with 5 ml of medium plus 0.48 mg/ml G418 (active). Selection was allowed to proceed for 10–14 days. Medium was changed as necessary during the selection process. Macroscopic colonies were stained with crystal violet and counted.

Retroviral RNA for slot blot analysis of retrovirus production was extracted from media conditioned for 24 h by producer cells. Each sample of medium (9 ml) was centrifuged for 90 min at 45,000 rpm in a 50Ti rotor. RNA was extracted from the pellet by the method of Berger and Birkenmeier,[25] except that the NP-40 lysis step was omitted. Replication-competent or wild-type virus production was assayed directly on D56 cells by the method of Bassen *et al.*[26] Alternatively, serial 10-fold dilutions of media conditioned by producer cells were added to NIH 3T3 cells seeded onto 6-cm plates as described above in the presence of 6 μg/ml Polybrene. The cells were maintained for 14 days to allow wild-type virus to spread in the culture. On day 14, 1 ml of the media from each culture was assayed directly on D56 cells as described.

Polymerase Chain Reaction (PCR) Analysis

A 335-bp sequence specific for the N2 provirus was amplified using the following primers: left hand primer, 5'-GGACCTTGCACAGATAGCGT-3' (anneals to the *neo*^{r} gene); right hand primer, 5'-CTGTTCCTGACCTTGATGTG-3' (anneals to the residual envelope coding sequences in the provirus). Samples of DNA (500 ng) were amplified according to the specifications of the manufacturer (U.S. Biochemical), except that 1 μl of [α-^{32}P]dCTP (800 mCi/mmol; Amersham) was added to each

reaction. Analysis was performed for 20 cycles with an annealing temperature of 52°C. The amplified products were visualized by autoradiography.

RESULTS

Prestimulation and Prolonged Co-culture Increase Gene Transfer Efficiency

Infection protocols of varying length were tested for their effect on gene transfer efficiency, using the ψβS1 virus and infection conditions we had previously shown to be optimal.[5] Four months post-transplantation, none of 14 mice reconstituted with cells exposed directly to producer cells contained the human β-globin gene. In

```
                                                                  # Positive
                                                                  at 3 months
        Co-cultivation
        0xxxxxxxxxxxxxx>Inject into                                  0/14
            on ψβS1     recipient mice

        Prestimulation Co-cultivation
        0-------------->xxxxxxxxxxxxxx>Inject into                   4/10
         (IL-3 and IL-6)   on ψβS1     recipient mice

                   Prestimulation      Co-cultivation
        0----------------------------->xxxxxxxxxxxxxx>Inject into   4/9
                   (IL-3 and IL-6)        on ψβS1     recipient mice

+---------+-------------+-------------+-------------+-----------------
5-FU   Marrow        48 hrs        96 hrs        144 hrs
(d-2)  Harvest
```

FIGURE 1. Schematic representation of three protocols for retrovirus-mediated gene transfer of the human β-globin retrovirus to murine pluripotent hematopoietic stem cells. All donor cells were from a single pool of cells isolated from female C57BL/6J mice treated with 5-FU 48 h prior to harvest. The length of prestimulation (---) and the length of the co-culture (xxx) are shown. The number of animals containing the human β-globin provirus (# positive) three months post-transplantation vs. the total number of animals in each group is shown at the *right.*

contrast, 4 of 10, and 4 of 9 mice reconstituted with cells prestimulated for 48 and 96 h, respectively, prior to exposure to retrovirus producer cells contained the human β-globin gene (FIG. 1).

Generation of a High-Titer Producer Cell Line

Amphotropic retroviruses can infect cells from a wide variety of species, including mice and primates.[18] A pA317 cell line producing 5×10^6 infectious particles/ml of the N2[20] virus was co-transfected with plasmids containing the gibbon IL-3, human IL-6, and hygromycin resistance genes. A hygromycin-resistant population, designated N263, was isolated which produced 10 U/ml gibbon IL-3, 50 U/ml human IL-6, and 5×10^6 CFU/ml. To increase recombinant retrovirus production and to minimize

the generation of replication-competent virus, amphotropic N263 cells were co-cultivated with ecotropic GP+E-86[19] cells for 14 days. The pA317 (parental cell line of N236) and GP+E-86 cell lines require two and three specific recombination events, respectively, to generate replication-competent virus.[18,19] On day 17, medium conditioned by the mixed pool of producer cells contained 1×10^9 CFU/ml (TABLE 1: Experiment 1) as assayed on NIH 3T3 cells (which detect both amphotropic and ecotropic virus). Individual amphotropic clones derived from the parental N263 cells were isolated from this mixed pool by selection in hygromycin. Four of 12 clones analyzed produced more than 1×10^9 *neo*^{r} CFU/ml, and two of these produced $\geq 1 \times 10^{10}$ CFU/ml as assayed on HeLa cells (which detect amphotropic virus only).

Characterization of the High-Titer Cell Line

One high-titer amphotropic producer clone (designated N263A2) was identified which secreted ca. 10 U/ml gibbon IL-3, 50 U/ml human IL-6 and 2×10^{10} CFU/ml. Southern Blot analysis of DNA from N263A2, N263, and NIH 3T3 cells with *Sst* I revealed that the parental N263 cell line had a 3.2-kb band representing a single unrearranged copy of the provirus (FIG. 2A and data not shown), while the N263A2 cell line contained approximately 20 unrearranged copies of the N2 genome. Increased production of recombinant retrovirus was documented by analysis of retroviral RNA extracted from medium conditioned for 24 h by the parental N263 and N263A2 cell lines. Serial 10-fold dilutions of this RNA were analyzed on slot blots with a *neo*^{r} probe. Comparison of the slot intensities demonstrated that the N263A2 cell line produced approximately 1000-fold more retroviral RNA than did the parental N263 cell line (FIG. 2B).

Replication-competent virus production by N263 and N263A2 cells was measured assayed by mobilization of a sarcoma virus genome in D-56 cells, resulting in plaque formation.[26] Direct infection of D-56 cells with N263- and N263A2-conditioned media revealed <1 and $<10^3$ replication-competent virus/ml, respectively. In a more sensitive (indirect) assay, NIH 3T3 cells were infected with dilutions of media conditioned by N263 or N263A2 cells, after which they were passaged for 14 days to allow helper virus to spread and amplify in the cultures. Medium conditioned by these 3T3 cells was then assayed directly for replication-competent virus as above. The greatest dilution of N263- and N263A2-conditioned media that contained replication-competent virus was 10^{-1} and 10^{-4}, respectively (TABLE 1).

High Retrovirus Titer Increases Gene Transfer to Murine Stem Cells

To test the effects of extremely high concentrations of recombinant virus on gene transfer efficiency, bone marrow cells isolated from mice treated 48 h previously with 5-FU were co-cultivated with N263 or N263A2 cells for 6 days in the presence of 200 U/ml murine IL-3. Because both the N263 and N263A2 cell lines secrete approximately 50 units of human IL-6 per milliliter of conditioned medium, these conditions are nearly identical to those we have previously shown to be adequate for gene transfer into murine stem cells (FIG. 1 and Ref. 5). Three months post-transplant, DNA extracted from the peripheral blood and bone marrow of these animals was analyzed by PCR for the presence of the N2 provirus. Only two of 13 mice reconstituted with cells co-cultivated with N263 cells were positive for the provirus (FIG. 3A). In contrast, 10/12 mice reconstituted with cells co-cultivated with N263A2 cells were positive for the provirus (FIG. 3B). Dilution experiments and Southern

TABLE 1. High-Titer Producer Cell Lines

Experiment No.	Cell Line: Amphotropic Producer	Cell Line: Ecotropic Producer	Titer of Pool (CFU/ml)	Clone	Titer of Recombinant Virus (CFU/ml)	Titer of Replication-Competent Virus (CFU/ml)[a]: Direct	Titer of Replication-Competent Virus (CFU/ml)[a]: Indirect
1	N263[b]	GP+E-86	1×10^9	A1	6×10^9	$< 10^5$	ND
				A2	2×10^{10}	$< 10^3$	$> 10^4$; $< 10^5$[c]
				A3	1×10^{10}	$< 10^4$	ND
				A4	3×10^9	$< 10^3$	ND
2	pA317	ψβS1[d]	5×10^8	E3	6×10^8	$< 10^5$	ND
				E7	6×10^9	< 10	ND
				E8	8×10^8	$< 10^3$	ND
				E12	4×10^9	< 10	ND

[a]Titer of replication-competent virus was assessed by direct assay in D-56 cells and by indirect assay on D-56 cells after passage in NIH 3T3 cells. ND, not done.

[b]Produced 5×10^6 CFU/ml.

[c]Greatest dilution containing replication-competent virus was 10^{-4}.

[d]Produced 2×10^6 CFU/ml.

blot analysis showed that there were between 0.1 and 0.3 copies of the N2 provirus per genome in these animals (FIG. 3 and data not shown).

High Retrovirus Titer Permits Gene Transfer to Rhesus Monkey Stem Cells

Bone marrow cells isolated from rhesus monkeys were co-cultivated with N263 or N263A2 cells for 6 days. The donor animals received two doses of total body irradiation (500 rads), separated by 24 h, prior to reinfusion of the infected cells. After the bone marrow had regenerated, samples of DNA extracted from peripheral blood cells were analyzed for the presence of the N2 provirus by PCR. No evidence of gene transfer was detected in three monkeys transplanted with cells co-cultured with N263 cells (FIG. 4, lanes 13 and 14, and data not shown). In contrast, the N2

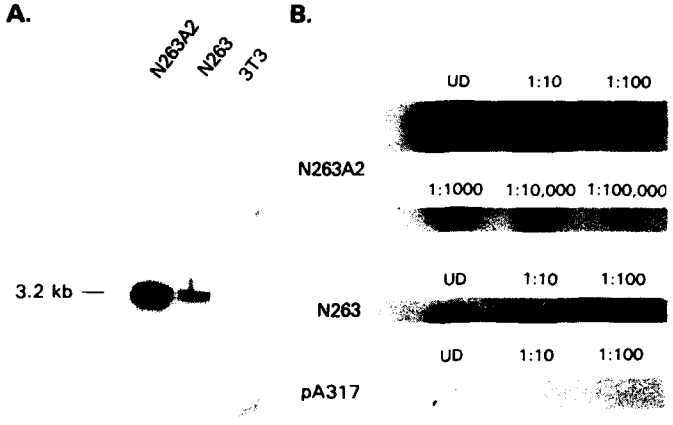

FIGURE 2. (**A**) Southern blot analysis of the N263 and N263A2 cell lines. 10 μg of DNA was digested with *Sst* I (which cuts once in each proviral LTR), resolved on a 1% agarose gel, blotted, and probed with a *neo*^{r} probe. (**B**) Slot blot analysis of RNA extracted from media conditioned by the N263 and N263A2 cell lines. Serial 10-fold dilutions of RNA were blotted and probed with a *neo*^{r} probe. UD, undiluted.

provirus was detected in circulating cells at all points examined between 20 and 99 days post-transplantation in each of three monkeys transplanted with cells co-cultivated with N263A2 cells. Significantly, the N2 proviral genome was detected in purified peripheral blood neutrophils, excluding the possibility that the signal was derived from infected lymphocytes that had survived in the circulation. One of the recipients developed renal failure secondary to a catheter infection and was sacrificed at 99 days post-transplantation. The N2 provirus was detected in DNA extracted from the peripheral blood, bone marrow, and spleen of this animal. Dilution experiments indicated that the fraction of blood or bone marrow cells containing the provirus was approximately 1% of the total in all three positive monkeys (FIG. 4).

All six of the animals infused with co-cultured bone marrow cells showed delayed reconstitution relative to animals transplanted with unmanipulated marrow cells

(TABLE 2). The average time between transplantation and when the neutrophil count reached 1000/mm^3 in our transplanted animals was 20 days as opposed to 11 days in previous studies.[24] This occurred despite the administration of GM-CSF beginning 2 days after bone marrow infusion and continuing until a neutrophil count of 1000/mm^3 was achieved. Similar delays in reticulocyte and platelet formation were observed in all the animals (TABLE 2).

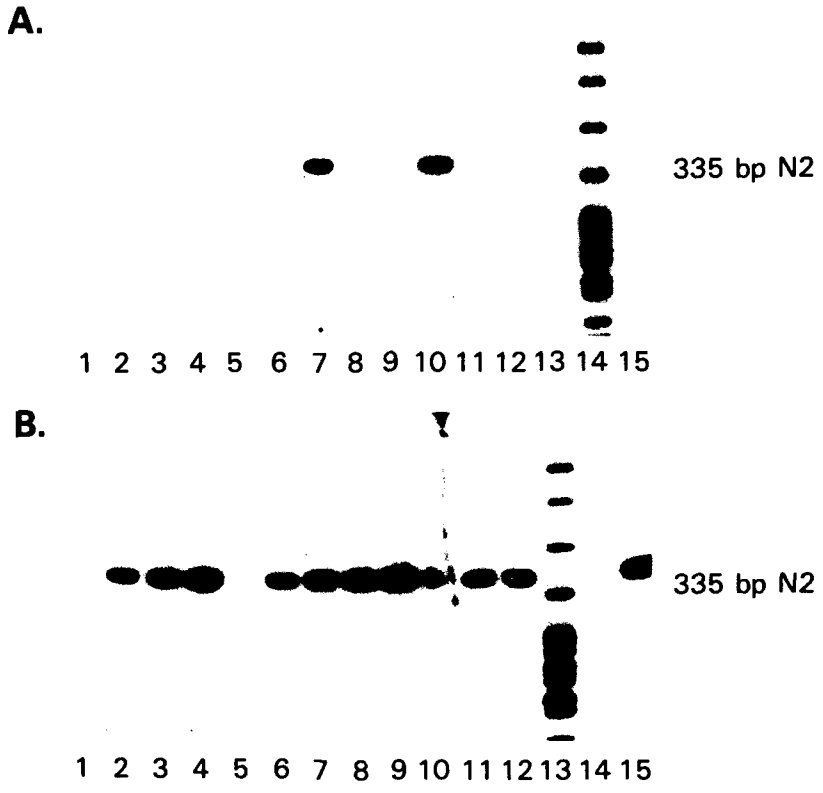

FIGURE 3. (A) (**lanes 1–13**) PCR detection of the N2 provirus in DNA isolated from mice reconstituted with bone marrow cells co-cultured with N263 cells, (**lane 14**) markers, (**lane 15**) analysis of DNA isolated concurrently from a negative control animal. (**B**) (**lanes 1–12**) PCR detection of the N2 provirus in DNA isolated from mice reconstituted with bone marrow cells co-cultured with N263A2 cells, (**lane 13**) markers, (**lane 14**) analysis of DNA isolated concurrently from a negative control animal, (**lane 15**) 1:10 dilution of PCR products generated from N263 (single copy) cells. *Markers* (*Msp* I digest of pBR322) are, from top to bottom, 622, 527, 404, 309, 242, 238, 217, 201, 190, and 180 bp, respectively.

DISCUSSION

Our experiments demonstrate that recombinant retroviral vectors can be used to insert genes into primate hematopoietic stem cells. Three successive animals have exhibited evidence of gene transfer into cells capable of contributing to long-term hematopoietic reconstitution. In addition, a fourth consecutive animal has exhibited

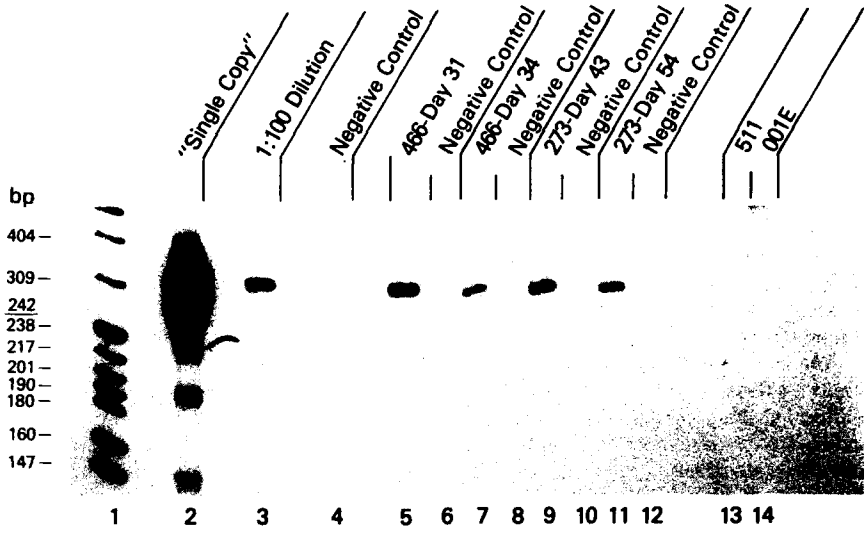

FIGURE 4. PCR detection of the N2 provirus in DNA isolated from rhesus monkeys reconstituted with bone marrow cells co-cultured with N263A2 or N263 cells. (**Lane 1**) markers, (**lane 2**) PCR products generated from DNA isolated from N263 cells, (**lane 3**) 1:100 dilution of PCR products generated from N263 cells, (**lane 4**) analysis of DNA isolated concurrently from 3T3 cells. (**Lanes 5, 7, 9, 11**) PCR analysis of DNA isolated from purified neutrophils (**lane 5**) or peripheral blood cells (**lanes 7, 9, 11**) from rhesus monkeys reconstituted with bone marrow co-cultured with N263A2 cells. (**Lanes 6, 8, 10, 12**) PCR analysis of DNA isolated concurrently from purified neutrophils (**lane 6**) or peripheral blood cells (**lanes 8, 10, 12**) from negative control animals. (**Lanes 13, 14**) PCR products generated from DNA isolated from peripheral blood of rhesus monkeys reconstituted with cells co-cultured with N263 cells.

evidence of gene transfer (unpublished observations). Three other large animal models have previously been utilized in gene transfer experiments. In a canine model, six dogs were reconstituted with autologous bone marrow co-cultured with retrovirus-producing cells, but only a very low level of drug-resistant progenitor cells

TABLE 2. Reconstitution after Gene Transfer to Rhesus Monkey Stem Cells

				Reconstitution Time (days)			
Animal No.	Producer Cell Line	Days in Culture	Cells Infused ($\times 10^9$)	To Neutrophil $>1000/mm^3$	To Platelet $>35{,}000$	To Reticulocyte $>2\%$	Provirus
001E	N263	2	1.5	13	22	23	−
0511	N263	6	1.0	18	26	19	−
0457	N263	4	1.8	8	23	22	−
273E	N263A2	6	1.2	19	29	29	+
0466	N263A2	6	1.3	22	—[b]	—[b]	+
0504[a]	N263A2	6	0.25	25	31	29	+
Average of 5 animals (a previous study)			1.2	9	18		

[a]This animal was pretreated with 5-FU (150 mg/kg).
[b]Persistant infections hampered the analysis of this animal.

were detected in three of the animals.[27] Drug-resistant progenitor cells were detected in the peripheral blood of 6/10 newborn lambs that had been infused, *in utero,* with autologous infected circulating blood cells. The provirus was detected in DNA from two of the lambs by PCR.[28] Gene transfer of the human adenosine deaminase (ADA) gene was attempted in a series of 20 cynomolgus or rhesus monkeys. None of the rhesus monkeys exhibited evidence of gene transfer. Two of the cynomolgus monkeys had detectable human ADA in ADA in peripheral blood cells at levels of 0.2% and 0.5%, respectively, of that of the endogenous monkey enzyme; and three other animals had very much lower levels of human ADA. The expression of human ADA was transient and could not be detected beyond 90 days post-transplantation in any of the animals studied. *In situ* hybridization and *in vitro* selection of drug-resistant cells suggested that gene transfer may have been to long-lived T lymphocytes in these animals.[29,30]

We have previously shown that in mice, the combination of IL-3 and IL-6 increases the number of CFU-S and the repopulating potential of bone marrow cells in liquid culture and enhances gene transfer to PHSC.[5] This combination of growth factors also permits murine gene transfer experiments to procede for up to six days *in vitro* without drastic reduction of repopulating potential. We have designed our primate gene transfer experiments based on these results. We co-cultivated our bone marrow cells with producer cells for 2–6 days, exposing them to retrovirus particles for much longer times than those used previous protocols. In the canine model, bone marrow cells were co-cultivated with producer cells for 24 h before reinfusion into the recipient.[27] In the fetal sheep model, circulating progenitor cells were cultured for 24 h in medium previously conditioned by producer cells for 24 h.[28] In previous primate studies either 24-h co-cultivation or 2–24 h of culture in producer cell–conditioned medium was used.[29,30] The producer cell lines used in the canine, fetal sheep, and previous primate studies had generated 5×10^6 to 2×10^7 retroviral particles per milliliter, a titer approximately 500-fold lower than that of the virus produced by N263A2 cells. In summary, prior protocols utilizing shorter culture periods and lower-titer viruses gave inconsistent infection, and in some cases the transduced cells may have been a population other than the hematopoietic cells capable of long-term reconstitution.

Our initial studies have identified several problems which must be resolved. All of our animals infused with bone marrow cells co-cultured with virus-producing cells required an average of 20 days to reach a neutrophil count of $1000/mm^3$. Previous studies have shown that by three weeks post-irradiation, significant regeneration of endogenous marrow cells can be expected,[31] even without the administration of GM-CSF. Therefore, our transplanted autologous cells are likely to be diluted by cells derived from stem cells that survived the irradiation protocol. Complications of the transplant procedure and prolonged pancytopenia necessitated sacrifice of three positive animals at 32, 54, and 99 days post-transplantation, limiting the period of observation with respect to the long-term persistence of the transferred gene. The optimum conditions to maintain and infect rhesus stem cells in culture must be defined.

Secondly, the large number of cells required to reconstitute a primate species, including humans makes the infection of unfractionated bone marrow cells an impractical approach for gene transfer into stem cells. Using current protocols, the $2–4 \times 10^{10}$ cells required to reconstitute a human would require infection in 30–60 l of medium. Murine hematopoietic stem cells have been purified that retain their ability to reconstitute irradiated animals.[32] Purification or partial purification of primate or human stem cells would reduce the number of cells to be infected while increasing the multiplicity of infection.

Finally, superinfection by co-culturing ecotropic and amphotropic producer cells has been shown to increase the production of virus by these cells. We have shown that individual clones producing exceptionally high numbers of retroviral particles can be isolated from a pool of producer cells. The role of replication-competent virus in increasing retroviral production by producer cells is not clear. All of the high-titer cell lines we have examined also produce replication-competent virus, but the level of helper virus production does not correlate with the titer of the recombinant virus. Amplification of replication-competent virus production by co-cultivation will inhibit superinfection by the recombinant virus if ecotropic replication-competent virus infects amphotropic producer cells, or vice versa. New packaging cell lines designed to prevent replication-competent virus formation have been developed and should be valuable in this context.[33,34]

SUMMARY

Retroviral-mediated gene transfer into pluripotent hematopoietic stem cells has been difficult to achieve in large animal models. We have compared several infection protocols in a murine model system and concluded that bone marrow can be maintained and infected *in vitro* for 2–6 days. We have also developed an amphotropic producer clone that generates greater than 10^{10} recombinant retroviral particles (CFU) per milliliter of culture medium. Autologous rhesus monkey bone marrow cells were co-cultured with either high- (2×10^{10} CFU/ml) or low- (5×10^{6} CFU/ml) titer producer clones for 4–6 days and reinfused into sublethally irradiated animals. The proviral genome was detected in blood and bone marrow cells from all three animals reconstituted with cells co-cultured with the high-titer producer cells. In contrast, three animals reconstituted with bone marrow co-cultured with the low-titer producer clone exhibited no evidence of gene transfer.

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Retroviral Vectors for the β-Globin Gene That Demonstrate Improved Titer and Expression[a]

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INTRODUCTION

Several criteria regarding the efficiency of gene transfer and the stringency of β-globin gene regulation must be met to determine if gene addition therapy for β-thalassemia and sickle cell anemia is feasible. These include transfer of the globin gene into enough pluripotent hematopoietic stem cells to ensure the presence of the gene for the life of the patient. In addition, the expression of the transferred β-globin gene must be confined to erythroid cells at the appropriate developmental stage, and the level of expression must be therapeutically useful. Finally, there must be a low probability of harmful effects on endogenous gene expression arising from insertion of the proviral genome. Replication-deficient retroviral vectors, which can infect a wide range of host cells, represent a promising option for transfer of a β-globin gene into bone marrow stem cells.[1] Human and mouse bone marrow cells have been infected successfully with retroviruses,[2-6] and recently, retroviral vectors were used to transfer the human β-globin gene into mouse bone marrow cells.[7,8] Mice reconstituted with the infected bone marrow showed long-term tissue-specific human β-globin protein and RNA expression. The infected marrow also resulted in long-term β-globin expression after transfer to additional mice in serial transplantation experiments, indicating that pluripotent hematopoietic stem cells rather than committed progenitor cells were infected.[7,8] But in both of these virus-mediated β-globin gene transfer experiments, the transduced human β-globin gene was expressed at a low level (1–2%) compared to that of the endogenous mouse β^{maj}-globin gene.[7,8] Our current work is focused on improving the efficiency of stem cell infections and on improving the level of expression from the virally transferred human β-globin gene.

Several studies have suggested that the titers of retrovirus stocks are directly related to the efficiency with which target cells can be infected.[1,9] We identified regions in the genome of the first β-globin vector we prepared, LNβ*HP,[6] that interfered with virus replication. By removing one such region from the promoter of the β-globin gene, we made the vector LNβ*SA, which exhibited a higher titer as well as an increased efficiency of infecting target cells.[8,9] We have now extended this process by removing replication-interfering regions from within the main body of the β-globin gene itself. Derivatives of the LNβ*SA vector have now been obtained that

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demonstrate 10-fold higher titers, with no alteration of β-globin expression properties.

We have also devoted considerable effort to raising the level of β-globin expression from integrated proviruses, since RNA expression from the "first generation" of β-globin retroviral vectors as assayed *in vivo*[7,8] has been below the level (about 10% of normal) which might be expected to have therapeutic utility in humans. Our approach has been to configure β-globin vectors with DNA fragments from the globin dominant control region (DCR)[10] or locus activation region (LAR).[11] Functional studies in transgenic mice revealed for the first time the importance for high-level β-globin gene expression of the 20–kilobase pair (kbp) region of the human β-like globin gene cluster which lies 5′ to the ε-globin gene.[10] This region is characterized by four developmentally stable DNase I–hypersensitive sites, which map 6.1, 10.9, 14.7, and 18.0 kbp 5′ of the ε-globin gene in human erythroid tissues or in mouse or human cell lines in which any of the β-like globin genes are expressed.[10–13] Regulatory events which occur initially in the hypersensitive site region may be necessary for the subsequent expression of the β-globin genes, as suggested by the finding in studies of somatic hybrids between human lymphocytes and murine erythroleukemia (MEL) cells that the four hypersensitive sites form prior to overt expression of the β-globin genes.[11] In addition, naturally occurring deletions that remove these hypersensitive sites leave the adjacent β-globin locus transcriptionally inactive.[14,15] It is now clear that the LAR can be reduced in size from 18 kbp to 2.5 kbp[16] or 6.5 kbp[17] and still retain the ability to direct high-level expression of a β-globin reporter gene after transfer to MEL cells.

Recently we added determinants for each of the hypersensitive sites to a β-globin retroviral vector to test how they would be propagated and whether they would influence expression.[18] Although all fragments from the LAR interfered with viral replication to varying degrees, we found that the individual hypersensitive sites differed in their ability to increase β-globin expression. Whereas the determinant for the −6.1 kbp hypersensitive site had no effect on β-globin RNA expression, determinants for the other three hypersensitive sites led to increased human β-globin expression. Determinants for several of the hypersensitive sites also activated transcription from the retroviral long terminal repeat (LTR), which raises the possibility that LAR–β-globin proviruses may induce expression from non-globin genes in erythroid cells. The implications of the data for the use of LAR–β-globin retrovirus vectors in gene therapy protocols are discussed.

MATERIALS AND METHODS

Cell Culture

Cells were grown in Dulbecco's modified Eagle medium supplemented with 10% calf serum (ψ2 cells) or 10% fetal bovine serum (all other cells). Cell lines included the adenine phosphoribosyltransferase–negative mutant of MEL cells,[6] the thymidine kinase–negative (TK^-) mutant of NIH 3T3 cells,[9] the ecotropic retrovirus packaging cell line ψ2,[19] and the amphotropic retrovirus packaging cell line PA317.[20] Murine erythroleukemia cells (MEL) were induced to differentiate either with 3 m*M* *N,N′*-hexamethylene-bisacetamide (HMBA; Sigma Chemical Co., St. Louis, MO.) or with 2% dimethylsulfoxide (DMSO) for 6 days. Cells expressing neomycin phosphotransferase (*neo*) activity were selected by addition of 1–1.5 mg/ml G418, of which about 50% was active. Clones of G418-resistant MEL cells were obtained by

plating in medium containing 2.5 mg/ml agar and 1–1.5 mg/ml G418. Colonies were removed after 12–20 days and then expanded in liquid cultures.

Construction of Retroviral Vectors

All retrovirus vectors discussed in this report were based on the vector LNβ*SA,[9] which contains both a *neo* gene under the control of the promoter and enhancer sequences of the LTR of the Moloney murine leukemia virus and a human β-globin gene. The human β-globin gene contains sequences from −615 bp (*Sph* I site) through +2482 bp (*Avr* II site) and is marked by a 6-bp insertion in the 5′ untranslated region.[6] The marked β-globin gene is inserted between the *neo* gene and the 3′ LTR, and it is transcribed in the opposite direction relative to transcription from the 5′ LTR. The LNβ*SΔ2A vector was derived from LNβ*SA by deletion of residues 582 through 823 (a 242-bp *Rsa* I fragment) from the second intron of the β-globin gene, with the β-globin cap site numbered as +1. The LNβ*SΔ3A vector was derived from LNβ*SA by deletion of residues 582 through 955 of the second intron of the β-globin gene. The LNβ*S2Δ3A vector had a fusion of the first exon to the second exon as well as the deletion in the second intron described for the LNβ*SΔ3A vector (FIG. 1). A map of the vector LNβ*SA is given in FIGURE 2. Cleavage sites for *Hin*d III and *Hpa* I, which were used for the construction of the LAR derivatives, are located 5′ of the human β-globin gene but before the 3′ LTR. Insertions of LAR fragments into the LNβ*SA vector were at the same site with respect to the human globin gene. Fragment 18s (s = small) which contained the −18 kbp hypersensitive site was a 487-bp *Hae* III fragment. Fragment 15s contained the −14.7 kbp hypersensitive site and was cloned into the vector as a 569-bp *Hin*d III/*Bal* I fragment. The −10.9 kbp site was cloned as a 2-kbp *Hin*d III fragment (fragment 11) or as a 1267-bp *Hin*d III/*Pvu* II fragment (fragment 11s). The −6.1 kbp hypersensitive site was cloned as a 1.4-kbp *Hin*d III fragment (fragment 6) or as a 761-bp *Rsa* I fragment (fragment 6s). Complete details on these fragments have been published.[18] The *Hin*d III sites of the fragments were cloned into the *Hin*d III site of the vector; the *Bal* I, *Pvu* II, or *Rsa* I sites were cloned into the *Hpa* I site of the vector. A construct which contained the −14.7 kbp hypersensitive site together with the −18.0 kbp hypersensitive site as a 1.0-kb *Hin*d III fragment (μ15,18) was derived from plasmids described elsewhere.[16] The μ15,18 fragment had *Hin*d III termini and was cloned into the viral *Hin*d III site.

Generation of Retroviruses and Infection of MEL Cells

Retroviruses were generated as described previously.[9] Briefly, supercoiled plasmids were transfected into the ecotropic packaging cell line, ψ2. Transiently expressed virus was harvested after 2 days and used to infect the amphotropic retrovirus packaging cell line PA317, which was plated in G418-containing medium. The structure of the integrated provirus in clonal retrovirus-producing cell lines was analyzed by digestion of producer line DNA with *Kpn* I, which cuts the provirus in each LTR, followed by Southern blot analysis with a *neo* probe. Viral titers were assayed by infection of NIH 3T3 TK$^-$ cells. MEL cells were infected with supernatant medium from amphotropic retrovirus-producing cell lines and grown in semisolid medium with G418; individual colonies were isolated, expanded, and, after digestion of their DNA with *Kpn* I as previously described,[6] analyzed by Southern blotting for the presence on an unrearranged provirus.

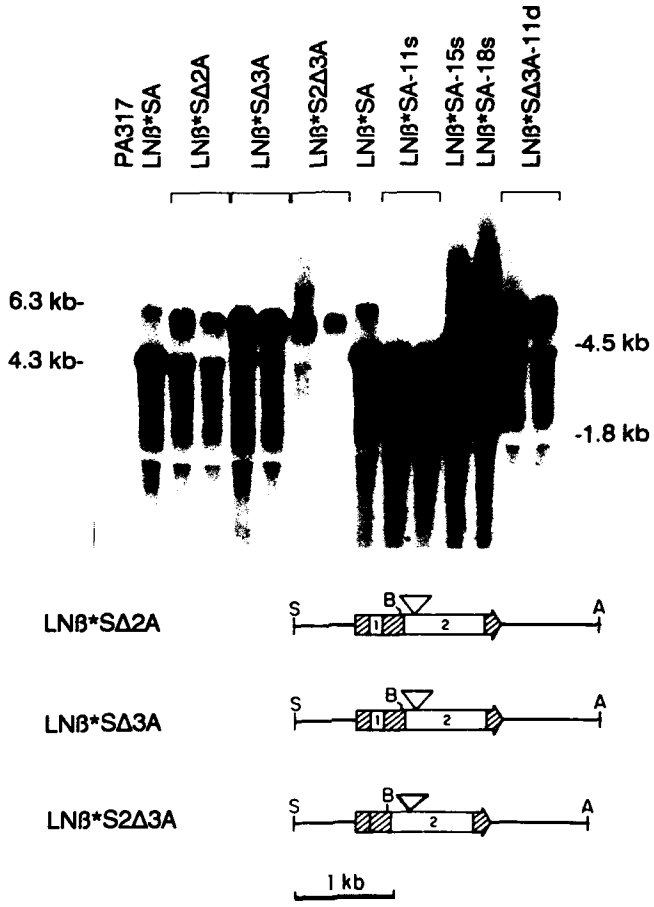

FIGURE 1. Analysis of proviral RNAs from different PA317 virus-producing clonal cell lines. (**Upper panel**) Northern blot of 10 μg of total cellular RNA from PA317 cells (negative control) or from PA317 virus-producing clones for each of the indicated constructions. Hybridization is to a *neo* probe. The sizes of the principal ribosomal RNAs are marked (4.5 kb, 1.8 kb). The full-length genomic RNA transcript from the LNβ*SA provirus migrates at 6.3 kb; subgenomic transcripts migrate at positions of 4.3 kb and smaller. Full-length proviral RNA from the derivatives of LNβ*SA with short deletions in the second globin intron migrate slightly faster than 6.3 kb. (**Lower panel**) The locations of the β-globin second intron deletions are indicated. S, B, and A: *Sph* I, *Bam*H I, and *Avr* II sites, respectively. Exons are shown with *hatched boxes*, and the first and second introns are *numbered*. Deletions are not drawn to scale. See MATERIALS AND METHODS for more details.

RNase Protection Analysis of Globin and neo *RNA*

RNA was prepared by lysis of the cells with guanidine isothiocyanate and subsequent selective LiCl precipitation.[21] Steady-state levels of human β-globin or mouse β^{maj}- and β^{min}-globin mRNA, as well as *neo* mRNA, were determined by

RNase protection assays as previously described.[6] The RNA probes that detect 5′ terminal fragments of human or mouse β-globin mRNA have been described in detail[6] and were labeled with [^{32}P]UTP. *Neo* mRNA was measured with an RNA probe which protected an internal fragment of the *neo* transcript from the *Nru* I site to the *Pst* I site, 119 nucleotides in length. The relative amounts of human and mouse β-globin mRNAs were quantitated by counting the radioactivity in the gel slices containing the protected RNA fragments after autoradiography of the gel; correction was made for background and for the number of uridine residues in the respective mouse and human probes. Total cellular RNA was isolated from PA317 packaging cell lines for Northern analysis as previously described.[9]

RESULTS

Alterations within the Second Intron Lead to Improved Titers

Previous studies from our laboratory on the β-globin retroviral vector LNβ*HP revealed that the introns of the globin gene were essential for production of steady-state β-globin mRNA after transfer into MEL cells.[6] During the course of this work we found that a vector for an intronless β-globin gene produced virus at nearly 100-fold higher titers than did vectors for the globin gene with both introns, which implied that sequences within the globin gene itself might be interfering with viral

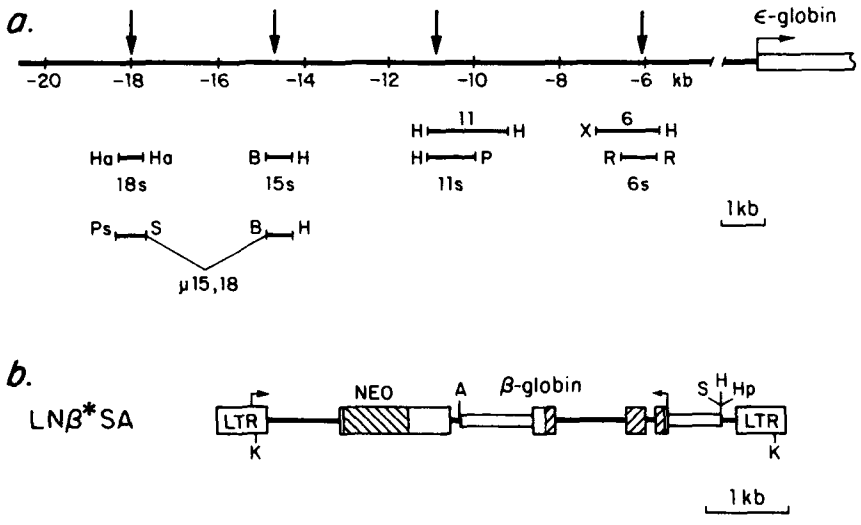

FIGURE 2. (a) Map of the LAR sequences used for retrovirus constructions. The locations of the DNase I-hypersensitive sites upstream of the ε-globin gene are indicated by *arrows*. The restriction fragments inserted in the viral vectors are shown. Restriction enzymes used were *Bal* I (B), *Hind* III (H), *Hae* III (Ha), *Pvu* II (P), *Pst* I (Ps), *Rsa* I (R), *Sph* I (S), and *Xba* I (X). **(b)** Map of the retrovirus LNβ*SA. The positions of the *neo* gene, its transcriptional start site in the LTR, and the reverse-orientation human β-globin gene within the virus are shown. The *hatched boxes* indicate coding, the *open boxes* non-coding, sequences. The locations of the introns in the globin gene are shown by *solid bars*. The cloning sites used for the insertion of LAR fragments (S, *Sph* I; H, *Hind* III; *Hp*, *Hpa* I), as well as the *Kpn* I sites (K) in the LTRs are shown.

replication. Support for this hypothesis was obtained by studying the structure of the RNAs transcribed from β-globin proviruses integrated in packaging cell lines or present in virions.[9] The amount of the full-length proviral transcript (6.3 kb), the only proviral RNA which was capable of transmitting the entire genome, was quite low relative to a group of lower molecular weight RNA species (4.3 kb and smaller). A minority of these latter RNA molecules were of a size which suggested that they originated in the proviral LTR and terminated within the promoter of the reverse-orientation β-globin gene, but the majority of the subgenomic RNAs apparently terminated within the second intron of the reverse-orientation β-globin gene. By removing a portion of the β-globin promoter, titers were improved and β-globin expression was not affected. The vector LNβ*SA was the result, in part, of this rearrangement. Unfortunately, the second intron could not be removed, at least in its entirety, since it was required for a normal steady-state level of β-globin mRNA expression.[6,9] We have now prepared derivatives of the LNβ*SA vector which contain deletions of portions of the second intron, to test whether the determinants which disrupt viral RNA replication could be separated from the element(s) which lead to normal stead-state levels of RNA.

We prepared derivatives of the LNβ*SA vector from which 242 bases (LNβ*SΔ2A) or 374 bases (LNβ*SΔ3A) of the β-globin second intron were removed. A third vector (LNβ*S2Δ3A) combined the larger deletion with a β-globin gene with no first intron, as shown in FIGURE 1. These deletions were chosen because the DNA in this portion of the intron has a high A+T content. As a result there are multiple occurrences of the AAUAAA motif on the non-coding strand of the β-globin gene, which is the same as the viral genomic RNA strand.[9] A Northern analysis of the RNAs transcribed from LNβ*SA and its three derivatives from separate PA317 virus producing cell lines is shown in FIGURE 1. PA317 virus-producing lines for the LNβ*SΔ2A, LNβ*SΔ3A, and LNβ*S2Δ3A viruses show abundant full-length viral transcripts, which migrate at slightly less than 6.3 kb. In contrast, the most abundant transcript for the LNβ*SA-producing cells migrates at about 4.3 kb, and the full-length transcript at 6.3 kb is much less abundant. As we previously showed, the 4.3-kb transcripts may be packaged into virions, but such virions are incapable of transmitting the complete LNβ*SA proviral genome.[9] The amounts of the full-length proviral transcripts are proportional to the viral titers (TABLE 1) exhibited by these packaging cell lines. Whereas the titer at which the LNβ*S2Δ2A virus could be produced was barely higher than that of the LNβ*SA, the LNβ*SΔ3A, and LNβ*S2Δ3A constructs showed titers 10-fold higher than that of the parental vector.

The possibility that the second intron deletions interfered with β-globin expression was tested in MEL cells. MEL cells are arrested at a late stage in erythroid development, but gene expression characteristic of terminal erythroid differentiation can be induced in culture.[22] After infection of MEL cells, a G418-resistant population was selected and treated with HMBA to induce erythroid differentiation. β*-globin mRNA expression from the LNβ*SΔ3A provirus, relative to the endogenous β^{maj}-globin expression, was the same as that from the LNβ*SA provirus, as shown in TABLE 2. Expression from the LNβ*S2Δ3A provirus was slightly lower (data not shown). The LNβ*SΔ3A vector will be used in the future to test the influence of LAR determinants on β-globin expression.

Production of β-Globin Viruses with LAR Fragments

Initially we ligated 1.3- or 2.0-kbp fragments specific for the hypersensitive sites located at 6.1 kbp (abbreviated as "6" in designations of the corresponding con-

TABLE 1. Isolation of Amphotropic Packaging Cell Lines Containing β-Globin Vectors

Vector	Insert Orientation[a]	Correct Structure[b]	Maximum Titer[c] (CFU/ml)
Parental *neo* virus[d]	—	>90%	1×10^7
LNβ*SA[d]	—	13/15	2×10^5
LNβ*SΔ2A	—	9/12	3×10^5
LNβ*SΔ3A	—	6/12	2×10^6
LNβ*S2Δ3A	—	9/10	2×10^6
LNβ*SA-6	R	1/12	5×10^2
LNβ*SA-6	F	0/40	—
LNβ*SA-11	F	0/40	—
LNβ*SA-6s	R	5/12	1×10^5
LNβ*SA-11s	R	2/44	1×10^3
LNβ*SA-15s	R	3/14	4×10^4
LNβ*SA-18s	R	1/6	2×10^4
LNβ*SAμ15,18	F	2/18	3×10^4

[a]Orientation of LAR-derived fragment relative to the β-globin gene: F, forward; R, reverse.
[b]Ratio of the number of PA317 producer lines containing the correct proviral structure (as determined by Southern blotting) to the number of PA317 lines analyzed.
[c]The highest titer on NIH 3T3 TK⁻ cells for the clones with the correct structure.
[d]Data previously reported.[9]

structs) or 10.9 kbp (abbreviated as "11") upstream of the ε-globin gene into the vector LNβ*SA (FIG. 2) to test if LAR fragments for single hypersensitive sites would be compatible with virus replication and would lead to elevated β-globin mRNA expression. Unfortunately, virus-producing cell lines made with the LNβ*SA-6

TABLE 2. Globin mRNA Induction and Relative Expression Levels in Infected MEL Cell Clones

Infecting Provirus	Colonies Analyzed (*n*)	mRNA Induction: Mouse β^{maj}-Globin[a]	mRNA Induction: Human β-Globin[b]	Ratio[c] Human β:Mouse β^{maj} Globin mRNA (%)
LNβ*SA	8	91 ± 114	6.3 ± 4.3	5.7 ± 3.8
LNβ*SA-6s	7	151 ± 60	6.0 ± 4.6	7.5 ± 7.7
LNβ*SA-11s	3	35 ± 25	290 ± 314	132 ± 157
LNβ*SA-15s	7	82 ± 51	12.3 ± 10.7	28 ± 18
LNβ*SA-18s	7	54 ± 30	6.0 ± 2.8	35 ± 26
LNβ*SA-μ15,18	13	141 ± 104	8.7 ± 6.7	15 ± 21
LNβ*SΔ3A	>100	29	12	5.1

[a]Ratio of mouse β^{maj}-globin RNA cpm in induced cells to mouse β^{maj}-globin RNA cpm in uninduced cells. For LNβ*SA;clone 1 (cl) actual cpm (corrected for background): uninduced cells, 313 cpm; induced cells, 16,875 cpm.
[b]Ratio of human β-globin RNA cpm in induced cells to human β-globin RNA cpm in uninduced cells. For LNβ*SA;cl actual cpm (corrected for background): uninduced cells, 1,343 cpm; induced cells, 3,467 cpm.
[c]Ratio of human β-globin RNA cpm (corrected for the 34 uridine residues in the human β*-globin RNA probe) to the mouse β^{maj}-globin RNA cpm (corrected for the 18 uridine residues in the mouse β^{maj}-globin RNA probe), expressed as a percentage ± one standard deviation. For LNβ*SA;cl human β-globin RNA: 3,467 ÷ 34 = 102; for mouse β^{maj}-globin RNA: 16,875 ÷ 18 = 937; (102 ÷ 937) × 100% = 11%.

and LNβ*SA-11 constructs showed large internal deletions, which encompassed not only the inserted fragment, but most, if not all, of the β-globin gene as well (TABLE 1). We next tested smaller LAR fragments (denoted with the suffix "s" in the constructs) in LNβ*SA. Fragments from the hypersensitive sites mapping 14.7 and 18 kb upstream of the ε-globin gene were abbreviated as "15" and "18", respectively. The LNβ*SA-6s, LNβ*SA-11s, LNβ*SA-15s, and LNβ*SA-18s viruses contained the indicated small LAR fragment in the opposite genomic orientation to the β-globin gene (FIG. 2). The LNβ*SA-μ15,18 virus carried a 1-kb fragment with determinants for both the 15 and 18 hypersensitive sites. Stable packaging cell lines were obtained with unrearranged proviruses for each of the small LAR fragments, although these were in the minority (TABLE 1). The titers observed for the LNβ*SA-LAR viruses ranged between 2×10^4 and 1×10^5 CFU/ml, which were adequate for infection of MEL cells and analysis of β-globin expression. FIGURE 1 shows that only low levels of full-length proviral RNAs were found in the PA317 virus-producing cell lines for the LNβ*SA-11s, LNβ*SA-15s and LNβ*SA-18s constructs, consistent with their low titers. Although the LNβ*SA-6s, LNβ*SA-15s, and LNβ*SA-18s proviral genomes were faithfully transmitted to MEL cells, as shown by Southern blot analysis, the LNβ*SA-11s, and LNβ*SA-μ15,18 packaging cell lines produced only 40–70% correct progeny virus, despite initially harboring unrearranged proviruses. FIGURE 1 shows that a high level of full-length proviral RNA is observed for a derivative of LNβ*SΔ3A, with a 150-bp fragment from the −10.9 kbp hypersensitive site region (LNβ*SΔ3A-11d), which should be contrasted with the pattern for the LNβ*SA-11s–producing cell lines, in which very little full-length RNA is detected by this Northern analysis.

Expression of the β-globin–LAR derivatives in MEL cells

Expression of the LAR–β-globin viruses was measured after infection of MEL cells. Individual MEL clones were isolated after culturing the cells in G418-containing semisolid medium. The structure of the integrated proviral DNA sequences and the pattern of proviral integrations were determined by Southern blotting for all cell lines. Only those G418-resistant MEL cell lines which carried single copies of the unrearranged provirus and which showed a unique site of integration were included in the analysis of β-globin expression.

Steady-state levels of human β-globin and mouse β^{maj}-globin mRNA were measured by RNase protection assays in RNA samples from MEL cells which were infected with the control LNβ*SA virus or its 6s, 11s, 15s, 18s, or μ15,18 derivatives. FIGURE 3 shows the pattern of correctly initiated human and mouse β^{maj}-globin mRNA from representative clonal MEL cell lines. After 6 days of treatment with DMSO or HMBA, induction of mouse β^{maj}-globin mRNA was consistently high. The amount of human β-globin mRNA after induction, expressed as a percentage of the mouse β^{maj}-globin mRNA in the same sample, is given in TABLE 2 for the cell lines shown in FIGURE 3 along with data from some additional cell lines. The amount of human or mouse β-globin mRNA after induction relative to the uninduced level, which is a measure of the extent of induction, is also presented for each clone in TABLE 2. Due in part to a low level of expression before induction, human β-globin mRNA induction was lower on average than the induction observed for the mouse β^{maj}-globin mRNA.

The parental LNβ*SA virus directed expression of the human β-globin mRNA in induced MEL cells at levels between 1% and 11% (averaging 5.7%) of the endogenous mouse β^{maj}-globin mRNA in eight cell lines (FIG. 3a and TABLE 2). Similar

levels of 2–24% (averaging 7.5%) were observed in MEL clones infected with viruses which contained the 6s fragment (FIG. 3b and TABLE 2). In contrast, the LNβ*SA-11s provirus expressed human β-globin mRNA at 10–310% (averaging 132%) of the endogenous level (FIG. 3f and TABLE 2). Proviruses which contained individual 15s or 18s fragments expressed human β-globin mRNA at steady-state levels between 5.8% and 55% (averaging 28%) or between 10% and 75% (averaging 35%), respectively, of the endogenous levels (FIGS. 3c, d and TABLE 2). Suprisingly, the LNβ*SA-μ15,18 virus, which carried determinants for both of these hypersensitive sites, directed human β-globin mRNA expression which ranged from 0.8% to 79% (averaging 15%) of the endogenous level (FIG. 3e and TABLE 2). The ratios of human to mouse β-globin RNA summarized in TABLE 2 represent conservative estimates of expression from the virally transferred globin genes, since no correction was made for the mouse β-globin gene copy number, which may have been higher than one per cell.

We also investigated whether DNase I–hypersensitive sites would be formed on the LAR fragments in the chromatin of the infected MEL cell lines. DNase I–hypersensitive sites formed on the 15s and 18s fragments present in their respective integrated proviruses. The 6s fragment also directed the formation of a hypersensitive site, although this LAR fragment had little if any effect on β-globin expression. MEL cells infected with the LNβ*SA-μ15,18 provirus showed only one hypersensitive site, which mapped to the 18s portion of the μ15,18 fragment. Hypersensitive sites which mapped to the 3′ LTR of the integrated provirus and to the promoter of the human β-globin gene were also present.[18]

LAR Effects on Non-Globin Gene Expression

Since the retroviral vectors used in this study transduced the *neo* gene as well as the human β-globin gene, we measured *neo* mRNA levels to determine if LAR fragments would influence transcription from the non-erythroid moloney murine leukemia virus (MoMLV) promoter. In a previous study of a retroviral vector (LNβ*HP) similar to the parental vector used here (LNβ*SA), *neo* mRNA levels in clonal MEL cell lines were low and did not always increase after induction.[6] A similar pattern of *neo* gene expression was observed for several LNβ*SA-infected MEL clones, as shown in FIGURE 4. In contrast, *neo* mRNA levels increased dramatically in response to induction in the MEL clonal lines which had been infected with the 6s, 15s, and 18s derivatives of LNβ*SA. The steady-state *neo* mRNA level increased 5- to 50-fold in the LAR-derivatives of the LNβ*SA virus (TABLE 3). The degree of induction was similar regardless of which LAR fragment was present in the provirus. The more stringent erythroid-like regulation of *neo* mRNA expression exhibited by the LAR–β-globin viruses is interesting in view of the inability of the 6s fragment to raise human β-globin expression in the same construct.

DISCUSSION

We prepared retrovirus vectors with the human β-globin gene along with the determinants for individual hypersensitive sites from the locus activation region (LAR) of the human β-like globin gene cluster in an attempt to identify regions of the LAR which would be stably propagated as a retrovirus and would lead to increased globin gene expression. In a parallel research effort, we found that the titer

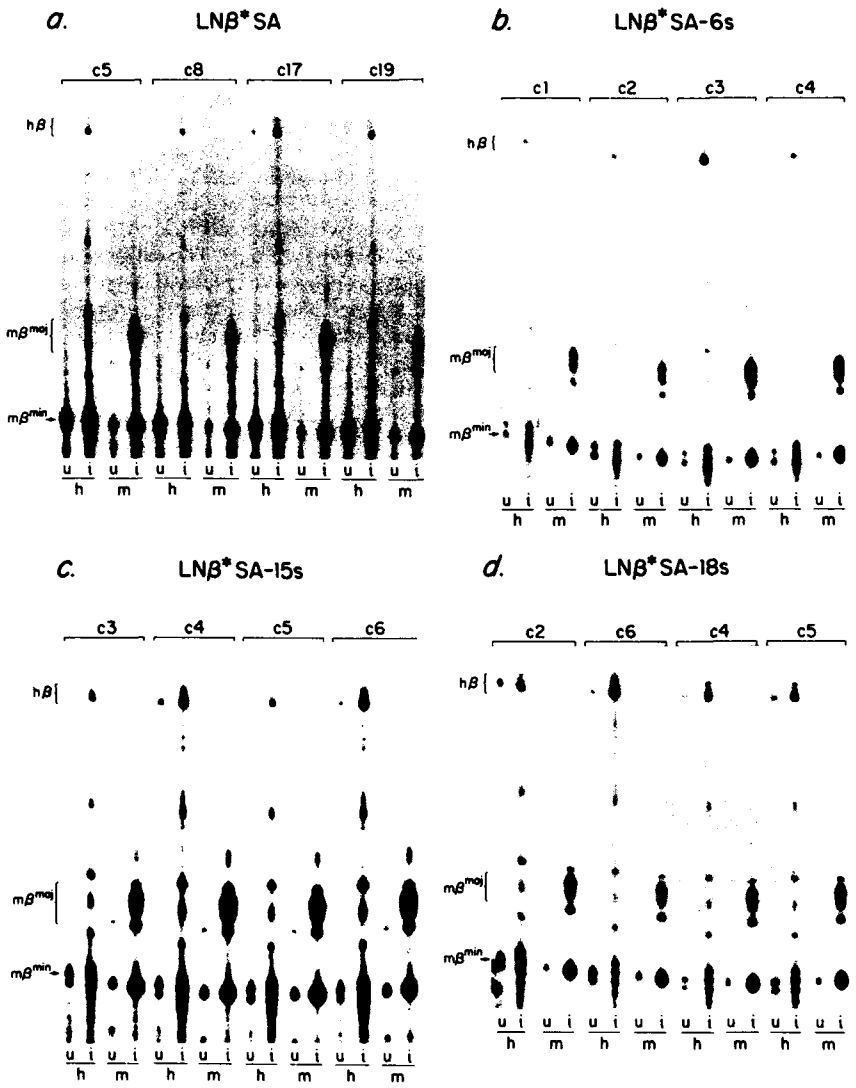

FIGURE 3. Expression of human β-globin mRNA in infected MEL cells. The levels of human and mouse β-globin mRNA were determined by an RNase protection assay. 3 μg of RNA from uninduced (u) or induced (i) clonal lines of MEL cells infected with (**a**) LNβ*SA, (**b**) LNβ*SA-6s, (**c**) LNβ*SA-15s, (**d**) LNβ*SA-18s, (**e**) LNβ*SA-μ15,18, or (**f**) LNβ*SA-11s viruses were hybridized with uniformly labeled RNA probes spanning the human (h) or mouse (m) β-globin mRNA capsites. The predominant fragment protected by the human β-globin probe was 138 nt, corresponding to correctly initiated transcripts (hβ). The mouse β^{maj}-globin probe protected fragments of about 60 nt derived from mouse β^{maj}-globin mRNA (β^{maj}) and 43 nt derived from mouse β^{min}-globin mRNA (β^{min}).

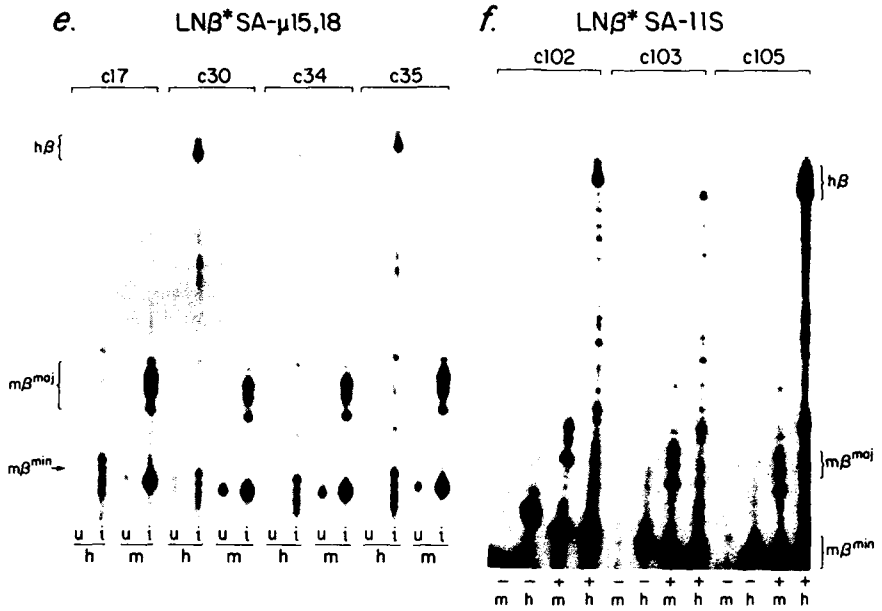

FIGURE 3. (continued).

at which the basic β-globin vector was produced could be increased over 10-fold, with no loss in β-globin expression, by removing a portion of the β-globin second intron. As this work continues, the most potent LAR fragments will be added to the improved vector, and tested *in vivo*.

After confirming the presence of single proviral insertions at unique positions, we studied RNA expression from a set of LAR–β-globin vectors in clonal erythroid cell lines like those which might be expected to result after retrovirus infection of hematopoietic stem cells.[18] One vector (LNβ*SA-11s) directed human β-globin

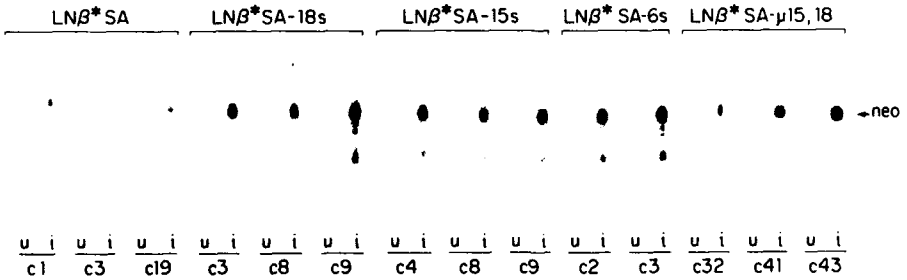

FIGURE 4. Effect of LAR sequences on *neo* gene expression. The levels of neomycin phosphotransferase mRNA in several uninduced (u) and induced (i) MEL cell clones infected with LNβ*SA, LNβ*SA-18s, LNβ*SA-15s, LNβ*SA-6s, and LNβ*SA-μ15,18 were assayed by RNase protection. 3 μg of RNA was analyzed after hybridization to a uniformly labeled RNA fragment containing sequences of the 3′ untranslated region of the *neo* gene.

mRNA expression in a small group of clones at a level comparable to that of the endogenous mouse β^{maj}-globin mRNA level. Viruses with determinants for the hypersensitive sites which map 14.7 or 18 kbp 5′ to the ε-globin gene also increased the steady-state levels of human β-globin mRNA in infected, induced MEL cells an average 5- to 7-fold, to 24% or 35% of the level of the endogenous mouse β-globin mRNA, respectively, compared to the control vector (LNβ*SA), which expressed human β-globin mRNA at an average of 5.7% of the endogenous level. β-globin retrovirus vectors which lacked LAR sequences showed regulated expression of the human β-globin gene after transfer into MEL cells,[6,23–25] but at levels which averaged 5–10%, at most, of the endogenous β-globin gene expression. Recently, Ryan *et al.*[26] also reported that a fragment which contained the determinant for the 10.9 kbp hypersensitive site resulted in a high-level human β-globin expression in transgenic mice, in agreement with these results.

The ratio of human β-globin to mouse β^{maj}-globin RNA varied 10-fold for the

TABLE 3. *neo* Expression in Infected MEL Cell Lines

Provirus	Clone No.	*neo* mRNA Level (cpm)[a]		Induction Ratio
		Uninduced	Induced	
LNβ*SA	1	147	477	3.2
LNβ*SA	3	51	127	2.5
LNβ*SA	19	88	421	4.8
LNβ*SA-6s	2	169	1524	9.0
LNβ*SA-6s	3	106	1938	18.3
LNβ*SA-15s	4	233	1178	5.1
LNβ*SA-15s	8	104	842	8.1
LNβ*SA-15s	9	158	1194	7.6
LNβ*SA-18s	3	171	1178	6.9
LNβ*SA-18s	8	19	1009	53.1
LNβ*SA-18s	9	110	2343	21.3
LNβ*SA μ15,18	32	12	517	43.1
LNβ*SA μ15,18	41	259	1179	4.6
LNβ*SA μ15,18	43	83	1653	19.9

[a]Counts per minute (corrected for background) measured with the *neo* RNA protection probe: uninduced MEL cell clones or cells induced with DMSO for 6 days.

control LNβ*SA virus and from 7.5-fold to over 30-fold for its LAR derivatives. Although variations in the extent of erythroid induction may have contributed to these differences, much of the variation probably reflects effects of the neighboring DNA sequences on the transcription of the different proviral insertions (position effects). Thus, the attribute of position-independent expression, which was described for the original "minilocus" in transgenic mice[10] and more recently for a 6.5-kbp derivative as tested in transgenic mice or in large polyclonal populations of MEL cells,[17] may only be a property of all four hypersensitive sites when present in tandem arrays, or of LAR sequences not present in the fragments we tested. Strict position-independent expression has not been observed in studies of the LAR from other laboratories. Ryan *et al.*[26,27] studied constructs similar to those originally reported by Grosveld *et al.*[10] in transgenic mice and observed elevated expression of human α- or β-globin genes, but the pattern of expression was neither strictly copy-number dependent nor position independent. In other studies of stably transformed MEL

cells, the ratio of human β-globin to mouse β-globin mRNA expression varied 5- to 6-fold with 8-kbp or 2.5-kbp forms of the LAR which included all four DNase I–hypersensitive sites,[16] or over 16-fold with the original minilocus construct.[28]

Interestingly, the 1-kbp fragment with determinants for both the −18 and −14.7 hypersensitive sites had less influence on β-globin RNA expression (15% of the mouse β^{maj}-globin level) than did either of these sites tested individually. In contrast, the same hypersensitive-site determinants present on a larger, 2.3-kbp fragment cause a linked β-globin gene to be expressed at more than 40% of the endogenous mouse β^{maj}-globin level after transfer to MEL cells by electroporation.[16] Conceivably, determinants necessary for high-level expression may have been left out of the smaller construct; or the −14.7 and −18 hypersensitive sites may show negative cooperativity if placed too close together, or too close to the retroviral LTR. This reservation applies to the other LAR–β-globin constructs as well. LAR subfragments small enough to be passaged through the life cycle of the retrovirus may lack determinants which are essential for activation and enhancement of erythroid gene expression.

The differences in biological activity revealed for the individual fragments which determine the −18, −14.7, −10.9, and −6.1 kbp hypersensitive sites are consistent with the phenotype of a naturally occurring deletion that eliminates most of the LAR. In a form of $(\gamma\delta\beta)^0$-thalassemia described by Driscoll *et al.*,[29] DNA which includes the region containing the −18, −14.7, and −10.9 kbp hypersensitive sites is deleted from the β-like globin gene cluster, but the −6.1 kbp hypersensitive site and the 3′ sequences, including all the β-like globin genes, are intact. No expression from the β-like globin genes in *cis* to this deletion can be detected, which suggests that the primary determinants for activation of β-globin gene expression lie upstream of the −6.1 kbp site.

In vivo expression levels are the most relevant to the question of the utility of retrovirus-mediated gene therapy. In the blood of mice which reconstituted fully with LNβ*SA-infected bone marrow, the human-to-mouse globin mRNA ratio was 1.5%,[8] or about 4-fold lower than the ratio observed in MEL cells in the present study (5.7%). Similarly, if expression from the 11s, 15s, or 18s derivatives of the LNβ*SA virus, which was 30% or more of the endogenous level in MEL cells, were also to decline 4-fold *in vivo*, it would be close to or above the minimum necessary for therapeutic benefit (about 10% of normal). *In vivo* tests of the LNβ*SA-LAR vectors, now in progress, may help to answer these questions since other factors, such as red cell lifetime, will also influence the therapeutic utility of a given mRNA expression level. The action of the LAR-derived fragments in elevating MoMLV-*neo* expression after induction is a reminder of the potential for insertional activation of non-globin genes in erythroid cells, with potentially deleterious consequences from retroviral vectors of this type in gene addition protocols aimed at gene therapy. Future experiments will test new combinations of the vectors and LAR fragments described here in order to facilitate experiments *in vivo*.

SUMMARY

To study the feasibility of a therapy for thalassemia based on addition of a correctly functioning globin gene to bone marrow stem cells, we have developed retroviral vectors that can transfer the human β-globin gene into pluripotent hematopoietic stem cells of the mouse. Mice reconstituted with virus-infected bone marrow cells showed long-term tissue-specific expression of human β-globin RNA and protein. Recently, we have redesigned the retroviral vector to improve the

efficiency of stem cell infection and to raise the level of globin expression obtained from the virally transduced gene. Removal of a portion of the second intron of the β-globin gene resulted in the accumulation of a higher level of full-length viral RNA in retrovirus packaging cell lines, and these cell lines produced β-globin virus particles at substantially higher titers. Addition of fragments from the locus activation region (LAR) of the β-like globin gene cluster to the retroviral vectors increased β-globin expression in infected murine erythroleukemia (MEL) cells. Fragments from the −18 and −10.9 kbp DNase I–hypersensitive sites of the LAR increased human β-globin RNA levels to 35% and 132% of the endogenous mouse β^{maj}-globin RNA level, respectively. Increased expression was also found for neomycin phosphotransferase RNA, which was transcribed from the retroviral long terminal repeat (LTR), showing that the LAR fragments also activated expression from a nearby heterologous promoter. These results are discussed in the context of the efficacy and safety of gene therapy for chronic anemia in humans.

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A Psychosocial Perspective

Growing Up with Thalassemia, a Chronic Disorder

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INTRODUCTION

The impact of a chronic condition such as Cooley's anemia is such that effective intervention requires a biopsychosocial perspective to better understand the effect on human development. *Cooley's anemia, Mediterranean anemia,* and *thalassemia major* all refer to a severe, inherited form of anemia first described in 1925 by Dr. Thomas Benton Cooley, an American pediatrician.[1] The complications require medical treatment; otherwise the patient dies in early childhood as a result of overwhelming infection. Although blood transfusions may be used to treat the symptoms of anemia, patients who receive only blood transfusions eventually die from complications caused by deposits of excess iron in the heart muscle.[2] A combination of blood transfusions and treatment with an iron-chelating drug to remove excess iron has extended life expectancy into the early 20s.[a]

To develop a psychosocial perspective, medical knowledge is important. Yet, in working with the individual and family, conceptualizing requires knowledge of the total context in which the individual functions. This includes not only the medical, but also the social and psychological, aspects and the resultant dynamic of the interaction of these three component parts which formulate the illness dynamic (FIG. 1).[3] The illness dynamic determines how the individual and family will respond to medical regimes and provides clues as to what is needed to enable the individual to realize his/her potential and to enhance the quality of life for the family.

Another way of examining the psychosocial perspective is to conceptualize the inner forces, the outer forces, and the person's behavior in response to the interaction of these forces. (FIG. 2).[4] The inner forces for an individual or family may be feelings of inferiority, frustration, and anxiety. The negative outer forces may be school, social agencies and the medical care delivery system, resulting in negative behaviors such as acting out and aggression (FIG. 3). The inner force is divided into two sectors (FIG. 4)—the emotional (the individual's reactions) and the biological (the medical realities of the condition). The outer forces include the social institutions—such as family, community, place of employment; and educational, health and welfare agencies—and culture, which takes into consideration the powerful and important outer forces deriving from ethnicity, social class, and social role. These dimensions are important since thalassemia affects not only people of Mediterranean descent, but persons of Middle Eastern, Southeast Asian, and African descent.

[a] For further information about thalassemia, see Refs. 14–19.

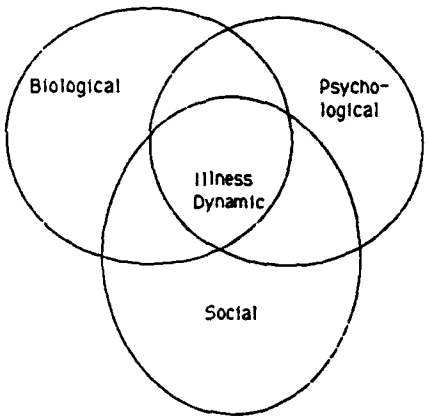

FIGURE 1. Biological (medical), social, and psychological component parts formulate the illness dynamic. (From Green.[3] Reprinted from *Mind and Body: The Psychology of Physical Illness* by permission of the American Psychiatric Press, Inc.)

PSYCHOSOCIAL IMPACT

The psychosocial impact of thalassemia on the development of patient and family is a continuous process throughout life. The implications of the disease are characterized and defined by the interactions of patient and family with the medical diagnosis

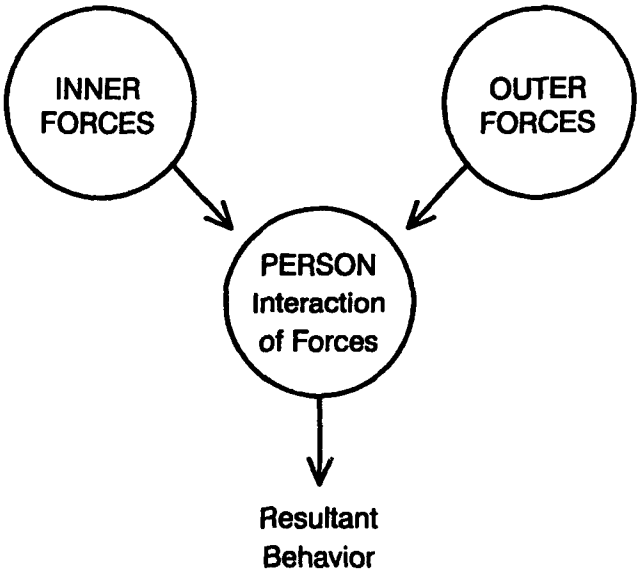

FIGURE 2. The psychosocial perspective may be examined by conceptualizing the inner and outer forces and the person's behavior in response to their interaction. (From Thackeray *et al.*[4] Reprinted from *Introduction to Mental Health: Field and Practice,* copyright 1979, by permission of Prentice-Hall, Inc.)

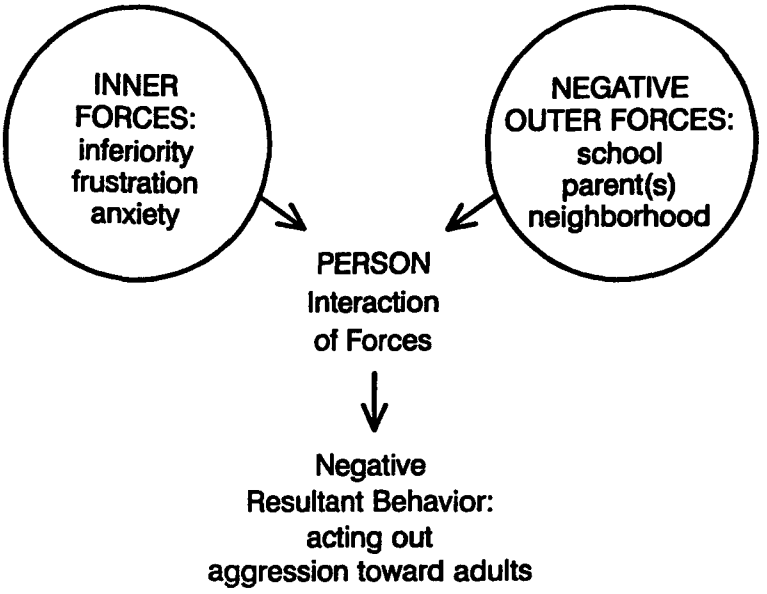

FIGURE 3. The interaction of inner and outer forces may result in negative behaviors. (From Thakeray *et al.*[4] Reprinted from *Introduction to Mental Health: Field and Practice,* copyright 1979, by permission of Prentice-Hall, Inc.)

and treatment. Prenatal and newborn screening has increased the likelihood of earlier knowledge of these interactions. When a diagnosis of a chronic genetic disorder is made, the reaction of the parents must be handled, as well as what this reaction might potentially do to the relationship between the child and his/her parents; the roles of the parents in dealing with the disorder must be considered; and the question of what resources will be necessary to cope with the condition must be addressed. Issues of denial, fear and anxiety, anger and hostility, withdrawal and depression are some of the feelings internalized by parents that could interfere with parenting or with following the recommended medical regime. No matter when the diagnosis occurs, there is a profound influence on the psychosocial domain. Problems are frequently intensified by the lack of adequate information and counseling, lack of support systems, and by the varying quality of medical facilities and the fragmentation of services.

Like other hereditary diseases, thalassemia may interfere with normal growth and development.[5] Parents are frequently overprotective. These attempts are further compounded by the parental feelings of guilt that surround the hereditary nature of the illness. Affected offsprings are often given an accurate prognosis of a shortened life span, which in turn affects motivation to achieve and encourages family members to foster dependency.

As the child with thalassemia moves from infancy to toddler and to preschool, the parents' reactions are frequently translated by the child into behaviors and attitudes of helplessness, dependency, lower self-esteem and/or self-worth.[6] Parents must often not only cope with the child's disease but also sacrifice the emotional and material needs of other siblings. This sometimes can result in a drain on the family

resources which may impact parents to the point that they perceive themselves as functioning ineffectively. For the child, the psychological impact may be devastating.

Experiences during infancy and early childhood can either promote or prevent the attainment of optimal development potential.[7] As the child moves out of the home and into the wider community, reinforcement of negative attitudes and behaviors may occur. The child may suffer teasing because of physical attributes. School attendance may be marked with absenteeism, leading to poor school performance and educational deficit. It is not uncommon that this may lead to social isolation and loneliness, which not only affect how the child feels about himself, but also affect his interactions with others. Through these formative years, the child may frequently experience limitations imposed by others but interpreted by the child as reflections of his/her own inadequacy. These communications and/or interpretations may be exhibited in the form of infantile depression, academic failure, social isolation, etc.

The stage of puberty and adolescence is an emotionally turbulent period for any child. For a child with a chronic condition, it is a stage of transition that encompasses the biological, psychological, and social aspects. Physical growth in adolescents with thalassemia commonly lags that of his/her peers. For the adolescent whose physical differences are apparent, the challenges of adolescence are often intensified. Having smaller physical attributes may further reinforce the adolescent's feeling of being different.[8] As the adolescent approaches high school, he/she may not feel adequate to compete, not only because of what may be a marginal academic record, but also because of how the adolescent feels about him/herself and the prognosis of the life line.

Psychosocial issues for the late adolescent and young adult thalassemia patient become focalized on issues of career, vocation, dating, and marriage.[9] The ramifications of these issues are compounded by how they are viewed by institutions such as school, human service agencies, and potential employers. Questions of dating and

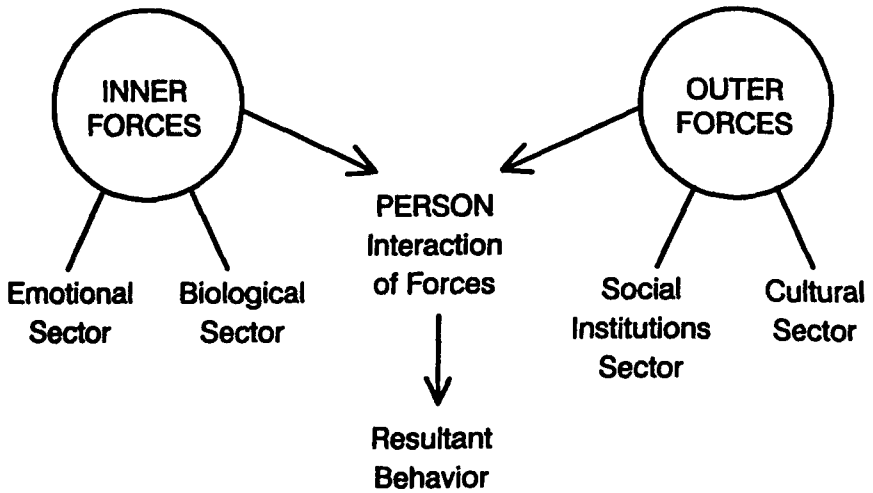

FIGURE 4. The inner and outer forces each consist of two sectors. (From Thackeray *et al.*[4] Reprinted from *Introduction to Mental Health: Field and Practice*, copyright 1979, by permission of Prentice-Hall, Inc.)

marriage are frequently marred by the status of the patients, by the fear of passing the condition on, and by uncertainty about their ability to earn a living. Issues of dependence and confinement may alter useful activities which may in turn affect family dynamics negatively.

Inherent through the life cycle is the stress on the child and family. These stressors include financial and emotional concerns and the efforts involved in finding medical care. The stress challenges the capacity to cope and adapt. In working with families, there is the necessity to understand how families function in order to know how to assist with coping and adaptation for all concerned.

ASSESSMENT AND INTERVENTION

Health care providers offering services to individuals and families where a chronic genetic condition exists should consider the following assumptions.[10] (1) There are predictable points of family stress. (2) Families vary in their level of tolerance for the patient's physical condition. (3) Families under stress tend to hold

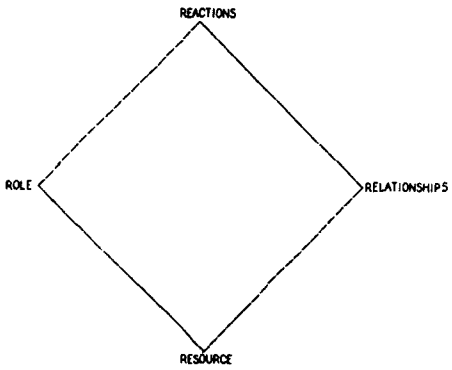

FIGURE 5. The four Rs for rapid psychosocial assessment, based on concepts presented by Doremus.[11]

to previously proved patterns of behavior whether they are effective or not. (4) Families usually go through a grief-loss process following the diagnosis of a disabling condition. (5) Families play a significant role in the encouragement or discouragement of the family member with a chronic illness to participate in particular therapies. (6) Families react to particular "illness behavior." (7) Many families have difficuly adjusting to the presence of a family member with a chronic physical illness because they either have incorrect or inadequate disease-related information. (8) Where there is a chronic illness, families must adjust to changes and expectations for each other. (9) A family's perception of the illness event has enormous influence upon their ability to cope.

Before intervening, there must be a psychosocial assessment. Doremus[11] offers a framework for rapid assessment, referring to the four Rs which can be applied to both the individual and the family. The four Rs are reaction, role, relationships, and resources (see FIG. 5). The reactions to the illness are the internal feelings and the behavior by the individual and family as a result of the diagnosis. The impact on parents, siblings, and the individual may cause new role demands which may not be within their capacity. Relationships may be altered, sometimes resulting in strained

family relationships which affect the ability of each family member to function effectively. Resources which the individual and family may need to function effectively make up the fourth R. This frequently includes counseling, financial assistance, and support. This framework offers a rapid assessment which is useful in a crisis situation such as hospitalization.

For ongoing work with individuals and families where a chronic condition exists, Christ[12] offers a more comprehensive framework for assessment for psychosocial intervention. She suggests that the dimensions of this framework include the following aspects:

1. The ecological framework of the treatment system. The features of this system may suggest what interventions can be aimed at the interface between the person and the environment. (The treatment system includes both the health care delivery system, where individuals and families may have to learn the roles and functions of members of the health care team, and the complexities of the treatment regimes, which can affect how the family functions and may increase rather than decrease stress.)
2. The expression of underlying psychopathology (e.g., a knowledge of psychopathology may be required to recognize clinical depression exhibited in reactions triggered by the diagnois, or the exacerbation of a prior existing mental illness).
3. A reactivation of underlying conflict. In families where difficulties appear to have been resolved, conflicts may re-emerge as a result of the patient's diagnosis.
4. Reactions to specific stress. The specific stress with Cooley's anemia may well be the treatment regime, the blood transfusions, and the discomfort. The types of stress reactions are divided into three categories: appropriate reaction, over-reaction, and under-reaction.
5. Dis-synchrony of coping among patients, family and health-care staff. The transactions among patients, families, staff members and the health care system—a transactional phenomena—can be uneven due to cultural and class differences where there are different perceptions of the meaning of terminology and procedures. At times this may result in goals, values, and coping styles which differ from those of the treatment team.

In the context of these frameworks for assessment, interventions can be determined for the family. Leahey and Wright[10] recommended the following criteria for determing the appropriate intervention:

1. A family member presents with a chronic illness that is having a detrimental impact upon the other family members.
2. Family members are contributing to the problems of an individual.
3. One member's improvement leads to symptoms or deterioration in another family member.
4. An emotional trauma or behavioral or physical problem with a child or adolescent develops in the context of the chronic illness.
5. A family member is diagnosed with a chronic illness.
6. A marked deterioration is observed in a family member's condition.
7. A chronically ill family member is moving from a hospital or rehabilitation center back to the community.
8. An important individual or family developmental milestone is missed or delayed.
9. The patient with the chronic illness has died.

There are three levels of intervention: the cognitive level, the behavioral level, and the affective level. Those interventions directed at the cognitive level include providing information about the chronic illness, suggestions about possible attitudes that the family may have, information about community resources, and help with decision making. At the behavioral level, families must be encouraged both not to make severe adjustments in their daily life and to find opportunities for respite. On the affective level, interventions are necessary if the family is overwhelmed and becomes disabled or if the family pretends that nothing has happened. Although in our perceptions, psychosocial interventions may seem appropriate, there are families where members may state that they do not wish to be involved in family sessions, or they may prefer to work with another health care professional in family treatment.

For the health-care provider offering psychosocial services, there are tasks and roles for both the family and the provider in facilitating the coping process.[13] The tasks for the family include maintainence of the motivation to cope with successive demands of the illness or disability, problem-solving activities to deal with the demands of the illness or disability and with other stress, management of painful emotions, and maintainence of optimal self-esteem—to permit coping efforts to be made and to maintain self-directiveness. The provider of service has the tasks of providing incentives and rewards for coping; providing instruction and coping skills individually and in groups; providing emotional support; providing information, time and space for effective coping; and providing opportunities for choice, decision-making and action.

The focus of the provider of psychosocial services must be both on the family and on the environment. For the role focused on the family, the provider must be a mobilizer, teacher, coach, enabler, and facilitator. With the environment, the provider must be a mobilizer, collaborator, mediator, organizer, facilitator, innovator, and advocate for the consumer.

Psychosocial assessments and interventions build on the existing strengths of individuals and families. This approach leads to an increasing degree of empowerment through the services offered. These include education, counseling, psychotherapy, mutual support and self-help, and support services, including community resources, such as vocational rehabilitation and mental health services. Additional services include crisis intervention, assertiveness training, transportation, legal help, and interventions with school, work, and the health-care delivery system.

In conclusion, then, chronic illness is a lifelong stressor which influences the psychosocial functioning of the individual and family. Different periods of the life cycle are vulnerable to specific psychosocial problems. The approaches taken in helping to deal with stress, coping, and adaptation affect the quality of life for families. There is increasingly the need for more research in the psychosocial area, to address the question of whether our interventions are effective. Feeling good about what we do as providers of psychosocial services is no longer an acceptable measure of our success; we must quantify and qualify what it is we do in order to more effectively relate it to the overall process of medical treatment. What we do has ramifications beyond the psychosocial; it has been shown that there is a correlation between health status and the support system that is in place for families dealing with a chronic condition.

SUMMARY

Thalassemia influences individual and family psychosocial functioning. Parental reaction to diagnosis affects the parent-child relationship, parents' roles, and family

resources for coping. A child frequently translates parental reactions into personal attitudes of dependency and lowered self-esteem. In coping with one child's disease, parents often sacrifice the needs of other siblings. Attainment of optimal development may be promoted or hindered. As the child's environment widens, negative attitudes may be reinforced. Poor school performance due to absenteeism, physical differences that lead to teasing, and limitations imposed by others may be interpreted by the child as personal inadequacy. Such feelings often persist through adolescence due to the individual's lag in physical growth, marginal academic record, poor self-concept, and/or pessimistic outlook on life. The central psychosocial issues of late adolescence and young adulthood—career, vocation, dating, marriage—are compounded by the perspective of institutions and potential employers. Different periods of the life cycle are vulnerable to specific psychosocial problems.

The burden of chronic illness is a heavy one for the patient and family. It is well for us not to forget just how heavy it can be. This hymn has particular significance for this population.[b]

I WON'T COMPLAIN

I've had some good days, I've had some hills to climb
I've had some weary days and weary nights
But when I look around and think things over
All of my good days outweigh my bad days
I won't complain.

Chorus

God has been good to me, He's been so good to me
More than this world could ever be, He's been so good to me
He drives my fears away, He turns my midnight into day
So, I'll say thank you Lord, I won't complain.

Sometimes my clouds hang low, I'd like to see them go
I've asked the question Lord, Why so much pain?
But He knows what's best for me, although my weary eyes can't see
So, I'll say thank you Lord, I won't complain.

Bishop Charles Watkins

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[b]Text as sung by the late Bishop Charles Watkins of the Pentecostal Assemblies of the World. Reproduced here with permission from Mrs. Jewel Watkins.

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Family Reactions and Relationships in Thalassemia

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The child or the older person with β-thalassemia is a chronically sick individual who suffers from a severe chronic hemolytic anemia necessitating treatment with transfusions for survival. His/her illness may be a source of chronic stress to him/herself, the parents, and the rest of the family. This situation results in the existence of various types of emotional reactions and behavioral patterns in the family, while the relationships of its members are also affected.

The literature on the subject of the psychological consequences of chronic illness on the affected children, adolescents, and young adults, and on their families is quite extensive. In particular, chronic illness in a child challenges the family at three levels:[1]

1. *The cognitive level.* The family has to learn about the cause of the illness, its course, the prognosis, likely complications and the treatment.
2. *The emotional level.* The family has to work through the anxiety and uncertainty caused by the illness, as well as their fears that the health of the sick member may further deteriorate and that he/she will die. Normally, the illness is experienced by the parents as a narcissistic trauma which concerns their creativity and potency, and this affects their emotional life and their behavior towards the sick child.
3. *The level of everyday routine* in the life of the family. The normal way of life and the routine of the family are affected by events such as visits to the doctor, medical examinations and interventions, and admissions to the hospital—events and practices, that is, which have to be incorporated into the daily routine and life of the family.

Thalassemia is one of those chronic illnesses whose prognosis has, since the mid-1970s, changed through the use of new treatment protocols, including frequent transfusions and the use of chelators to prevent hemosiderosis. The result is that the average lifespan of these individuals has increased. Adult patients now attend college, get married and, even, divorced.

The improvement in the prognosis of the illness has begun to affect the attitudes of those who have to deal with the thalassemic patient and the relationships within the patient's family, even if thalassemia is still regarded by many as a fatal illness. Moreover, hemoglobinathy control programs, through the use of training programs on health education and antenatal diagnosis, have reduced the number of births of children with β-thalassemia.[2] Nevertheless, it continues to constitute a problem for the medical and social services in a considerable number of countries (particularly those with inadequately organized services). Furthermore, the various complications from transfusions—hepatitis-B and more recently AIDS—and non-compliance with therapy are major hazards for these families.[2,3]

In this study we shall describe the relationships and family reactions of patients

with thalassemia. The observations are derived from the Department of Psychological Paediatrics of the "Aghia Sophia" Children's Hospital and, in particular, from the psychosocial support program for thalassemic patients followed up by the Thalassemia Unit of the 1st University Paediatric Clinic and the corresponding Unit of the National Health System at the "Aghia Sophia" Children's Hospital.

The total number of patients monitored is approximately 1,200. These come from both Athens and the provinces; their ages range from infants to 30 years. In particular, the summary observations which will be given are derived from the following individual projects:

1. A systematic study of 40 children suffering from β-thalassemia.
2. A systematic program of weekly meetings of groups of parents addressed to parents of patients with thalassemia.
3. Clinical observations from liaison-consultation psychiatry of the Department of Psychological Paediatrics in the β-thalassemia units of the "Aghia Sophia" Children's Hospital and at the center for vocational training of young people with chronic illnesses. A more detailed description has been given elsewhere.[4-6]

After presentation of the observations from these projects, I will present our conclusions and recommendations.

SUMMARY OF THE STUDY OF MENTAL DISTURBANCES AND INTELLIGENCE AMONG CHILDREN WITH β-THALASSEMIA

The study had the following objectives:

1. To investigate the existence of psychiatric disorders in children aged 7½ to 12 years who suffered from β-thalassemia.
2. To assess the personality, defence mechanisms, and anxiety displayed by these children.
3. To investigate the self-concepts of children with β-thalassemia.
4. To assess the mental capacity of these children.

Methodology

Sample

The experimental group consisted of 40 randomly selected thalassemic children (20 boys and 20 girls) aged 7½ to 12 years. The control groups used were children suffering from chronic (motor and sensory) non-fatal conditions or handicaps (21 children: 13 boys and 8 girls); and normal children matched for age, sex and socio-economic class (35 children: 15 boys and 20 girls).

Assessment Procedures

The method described by Rutter and Graham[7] was used to investigate individual disturbances of the children with β-thalassemia or with other chronic conditions. This method includes a semi-structured interview with the child and a semi-structured interview with the parents about the child.

Psychological assessment of the children was conducted with the use of the following tools: (*a*) WISC (Wechsler Intelligence Scale For Children), to test the intelligence of the children in all three groups; (*b*) the Rey Complex System for intelligence assessment;[8] (*c*) the TAT projective test for the assessment of death anxiety and defense mechanisms;[9] (*d*) the Piers-Harris or "The Way I Feel about Myself" Scale for assessing the self concepts.[10]

In addition, two questionnaires were drawn up for the purpose of the assessment to investigate certain parameters of the medical history of β-thalassemic children, as well as social and family characteristics and functioning.

Synopsis of the Results

Rate and Type of Psychiatric Disorder

Children with β-thalassemia display a high incidence of symptoms of psychiatric disorders, with neurosis and anxiety being the most frequent diagnosis, followed by

TABLE 1. Piers-Harris Scale: Comparison of β-Thalassemic Children with a Paired Control Group of Normal Children[a]

Factor	Mean		Standard Deviation		Min–Max	
	Thal.[b]	Normal	Thal.[b]	Normal	Thal.[b]	Normal
Behavior	12.97	13.60	2.491	2.061	4–16	7–17
Intellectual and school status	14.60	14.49	2.047	2.092	9–17	9–17
Physical appearance and attributes	10.46	10.09	2.343	1.946	2–16	5–14
Anxiety	10.20	11.00	2.541	2.401	3–14	5–14
Popularity	8.54	8.91	2.020	1.721	5–12	4–12
Happiness and satisfaction	9.23	9.17	1.165	1.272	5–10	7–14

[a]Multivariate analysis: TSQ = 5.74, $p = 0.5661$.
[b]Thal., β-thalassemic.

depression. More specifically, 42.2% of the thalassemic children and 31.2% of the control group (chronically sick children) were diagnosed as having a psychiatric disorder. It should be mentioned here that findings from epidemiological studies indicate that for chronically sick children the percent judged to be maladjusted on the basis of parental ratings varies between 22% and 44%, depending on the type and the severity of the illness.[11,12]

Assessment of Self-Concepts

The self-concepts of children with β-thalassemia were normal (TABLE 1). A possible explanation of this finding is that the children with β-thalassemia tend to develop compensatory mechanisms and coping strategies against the anxiety, insecurity, and uncertainty created by the chronic illness and its repercussions. These findings are in agreement with similar work regarding children with other chronic disorders (nephrosis, diabetes, cystic fibrosis).[13] However, children with β-thalassemia who have facial and other physical abnormalities tended to have negative self-

TABLE 2. Piers-Harris Scale: Comparison of β-Thalassemic Children without Somatic Abnormalities (A) with Those Having Somatic Abnormalities (B)[a]

Factor	Mean[b]		Standard Deviation		Min–Max		F-Ratio (1.38 df)	Univariate Analysis p
	A	B	A	B	A	B		
Behavior	13.09	10.63	2.234	3.503	6–16	4–15	6.16	0.0176
Intellectual and school status	14.66	13.88	2.010	2.295	9–17	9–17	0.92	0.3446
Physical appearance and attributes	10.19	11.00	2.494	1.195	2–16	9–13	0.79	0.3793
Anxiety	10.19	7.00	2.620	3.742	2–14	3–13	7.95	0.0076
Popularity	8.72	7.00	1.836	2.449	5–12	4–12	4.91	0.0328
Happiness and satisfaction	9.31	7.37	0.998	1.923	6–10	5–10	16.09	0.0003

[a]Hottelling's $T^2 = 21.145, p = 0.0171$.
[b]For A, $n = 32$; for B, $n = 8$.

concepts in the following factors of the Piers-Harris Scale: anxiety, popularity, and happiness and satisfaction (TABLE 2). We can assume that when a chronic disease causes visible abnormalities in the facial and body appearance, the child is affected and develops negative self-concepts with possible consequences in his/her emotional development and level of psychosocial functioning. Other factors may also play a role here, such as the innate vulnerability and the life experiences, as well as coping strategies, of the child.

Personality Assessment

The assessment of personality revealed that the defense mechanisms of denial and displacement were used more often, to a statistically significant degree, in the thalassemic children than in the children with chronic disorders (TABLES 3 and 4). The assumption is that this is due to the compensatory mechanisms used by the ego of the child. However, it should be mentioned that mechanisms of both denial and displacement, and especially the first, are maladaptive mechanisms and may reflect, among other things, the general atmosphere and attitude of the family towards the child with thalassemia. It is known that quite often there is an atmosphere of secrecy and silence in the family in relation to the disease.

TABLE 3. TAT Projective Test for Displacement: Comparison of β-Thalassemic Children with Children Having Other Chronic Disorders[a]

Value	Thal. $n1 = 16$	Chronic $n2 = 13$
Mean	3.25	1.077
Standard deviation	1.57	1.320
Sum of ranks	312	123

[a]Mann-Whitney U Test, two independent samples: $p < 0.05$. Thal., β-thalassemic; Chron., chronic disorders.

TABLE 4. TAT Projective Test for Denial: Comparison of β-Thalassemic Children with Children Having Other Chronic Disorders[a]

Value	Thal. $n1 = 16$	Chronic $n2 = 13$
Mean	2.437	1.154
Standard deviation	1.861	1.405
Sum of ranks	286	149

[a]Mann-Whitney U Test, two independent samples: $p < 0.05$. Thal., β-thalassemic; Chron., chronic disorders.

Intellectual Functioning (I. Q. G.)

The I. Q. level of the children with β-thalassemia was compared to that of the normal children (TABLE 5). The intelligence of children with β-thalassemia was within the normal range.

In order to investigate whether other factors may play a role in the cognitive development of the children, we compared thalassemic children with facial and body abnormalities to those without them; also compared the history of operations and the time that blood transfusions started—before or after the first year of life. We found no differences in I. Q. level between the various groups. Similar findings regarding the I. Q. level were found using the Rey complex system.

GROUP WORK WITH THE PARENTS OF CHILDREN AND ADOLESCENTS

Method and Sample

This work was addressed to parents of children with β-thalassemia. The initial sample consisted of 15 parents. The group was semi-closed. In the first year it was attended only by the mothers; after that the fathers started to come. The group was conducted by a psychiatric social worker and a pediatrician, under the supervision of a child psychiatrist. The meetings of the group took place initially every 15 days and afterwards every week. This group met for four years.

Observations from Work in the Group

Several clusters of psychological reactions and behavioral patterns were observed. There was depression in the parents, often masked or with overt symptomatology. Guilt accompanying depression was also present and was associated with self-blame. The self-blame was related to the hereditary nature of the disease and

TABLE 5. I.Q. Level: Comparison of β-Thalassemic Children with a Paired Control Group of Normal Children

Value	Thalassemic	Normal
Mean I.G.G. ± SD	115.03 ± 17.52	120.7 ± 15.13
Mean I.G.V. ± SD	119 ± 17.85	122.7 ± 13.94
Mean I.G.P. ± SD	108.8 ± 18.78	114.4 ± 20.33

the fact that the parents had passed it on to the child. It is of interest that the mothers felt more guilt than the fathers. This appears to bear a cultural connection to the state of relations between the sexes in Greece; but, above all, emotionally, it relates to the fact that the mother carried and gave birth to the sick child, regardless of the genetic logic of equal and joint contribution to the inheritance of thalassemia.

Death Anxiety

Death anxiety was an issue which often dominated the discussions, particularly when any of the children manifested even a relatively simple complication. It is interesting that although the prognosis of the disease has improved significantly, the parents still were greatly preoccupied with death and their attitudes had not changed to a significant degree. During the last 3–4 years, a very small number of patients were found to be HIV-positive due to blood transfusions. The possibility of infection has clearly influenced the parents, as well as the medical and nursing staff, with the result that there has been a recrudescence of death anxiety, even in families of patients who are seronegatives for HIV.

Denial of Problems and Feelings

Some of the parents found it very difficult to come to terms with the fact of their child's disease and denied it completely. This led them to behave in ways which did not facilitate the normal psychosocial development of the child. Some of these ways were

1. *Overprotective behavior,* with the result that the children remained immature and dependent upon the parents, with few friends and an inability to accept limits to their behavior.
2. *Excessive pressure on the sick child* expressed through great demands in the sphere of academic achievement or even in sporting activities.

The denial of the problem sometimes reached the point of the word "anemia" becoming taboo within the family. Everyone knew about it, but nobody talked about it. Each member of the family was isolated and alienated from the rest, particularly from the child with the problem, who needed to express his/her feelings, and to know what was wrong and why he/she had to have transfusions.

It should be said that sometimes this denial took the form of adaptive denial and the life of the family continued without being negatively affected by the disease. In these cases communication between the members of the family was not hindered and there was no masochistic preoccupation of the whole family with anemia.

The relationship of the couple was also affected. They were isolated and alienated from one another, and the child became the only point of communication between them. The latter fact, of course, places a heavy burden on the sick child, since he/she is not allowed to have his/her own living space. Furthermore, since the parents are overly preoccupied with the child with anemia, they often neglect the needs of their healthy child/children, who may become alienated from the life of the family. In Greece, the extended family may become involved, which leads to a vicious circle of relationships (FIG. 1).

Sometimes the relationship of the family with the neighborhood or with the immediate social environment is affected and the family is isolated socially, hiding

themselves and the sick child, particularly if he/she is pale or has other abnormalities in his/her external appearance. This denial is associated with the guilt of the parents. Social factors appear to play a part, since the parents feel that the sick child is proof of their inadequacy. Any negative attitude of the social environment towards the family also plays a significant role in reinforcing this behavior. The relationship of the child with the school environment is also influenced. The child may need to be absent and finds him/herself in a very difficult position when the messages received from the parents are that he/she should not talk about why he/she is absent or he/she is forced to tell lies. This is another expression of the conspiracy of silence.

Reactions to the Pronouncement of the Diagnosis

Another factor which appears to be able to color the life of the family from the beginning is the shock occasioned in the parents and the family by the discovery of the illness. Many mothers stated that all their expectations and hopes had been

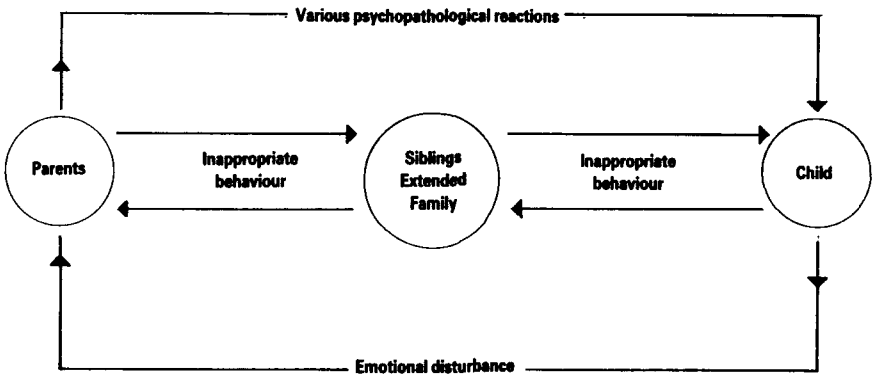

FIGURE 1. Vicious circle of the behavioral pattern of family relationships.

shattered and that they felt at a disadvantage, with frequent undervaluing of themselves and depression and guilt, which could influence the relationship with their child. Others reacted with anger against their own generation who had passed on the disease; often the anger is projected outwards and on to the doctors. One mother cursed Cooley for discovering the disease.

This period of shock is usually followed by a period resembling that in the children of grief described by Kübler-Ross,[14] which leads to the acceptance of the disease and a restructuring of the family life around it, its treatment and its management.

Adolescents' Processes and Family Reactions

I will add some more specific observations in connection with adolescents and young adults. First of all, the specific developmental problems and tasks of adolescence are both intensified and extended by the presence of thalassemia. Some of

these processes are the search for a new identity—including sexual identity—and the quest for independence and the consequent problems with authority figures and the relationships with the peer group.

Chronic illness is experienced by adolescents as a narcissistic trauma resulting in the mobilization of anxiety, particularly as regards their body. Furthermore, the privations, the restrictions, and the various medical procedures imposed on their bodies, as well as the changes brought about by their illness, generate various fantasies and conflicts focusing on their bodies.[15] In many thalassemic adolescents, puberty is delayed because of the underfunctioning of the endocrine glands as a result of iron deposition secondary to blood transfusion. Hormonal adjustment may be required to bring it on; such therapy is not compatible with his/her peer group. The delay in puberty is a further obstacle in the quest for sexual identity and body image, which has already been affected by the chronic illness. The quest for independence is also affected. Healthy conflicts of adolescents with their parents, which are entirely natural in their attempts to be different from them, are charged with anxiety, guilt, the overprotective attitude of the parents, or anger on the part of the adolescents when they discover that their parents are not divine beings who are going to save them from the disease.

The relationship of the chronically ill adolescents with the peer group can also be affected, since they feel different from the others who do not have the disease. It is our impression that adolescents and young adults, even if they do not in any way differ from their peers in their external appearance, continue to feel that they are different. This often leads these young people to seek the company of others who also have the illness, which can sometimes intensify the feeling that they are different.

All the above can of course result in the illness being used by both the parents and the adolescent as a focal point for all their conflicts and as an explanation of their difficulties. The outcome is that often the family is trapped in the vicious circle of guilt and conflicts, which, in effect, do not allow the adolescent to resolve the natural conflicts of his/her age. Depressive reactions in the family may also be observed; these are often accompanied by fear, guilt, ideas of death and feelings of powerlessness. The adolescent him/herself may show anxiety, depressive reactions and the feeling of not being understood, with desires for death or confrontation with the parents or the doctor—conditions which may manifest themselves either as noncompliance with the therapy or difficulties in social integration.

Natural Reactions of the Family

There are of course cases where the fact of the anemia, after the first shock resulting from the diagnosis, is handled by the family in a satisfactory manner. Communication between the members of the family is open, even on subjects connected with the anemia. Furthermore, the child's approach is within the natural framework. The needs of the parents or of the healthy children are not overlooked, while, at the same time, the parents' demands upon the sick child are realistic.

CONCLUSIONS AND RECOMMENDATIONS

It is obvious from what has been said up to now that thalassemia causes reactions of a variety of types in the members of the family and in the family as a whole, with

the result that the family relationships are affected; this may lead to a vicious circle of relationships.

Generally, it could be said that fairly often parents and children, particularly adolescents, use the illness as the focal point for all their conflicts and as an explanation of their difficulties. Furthermore, our impression is that a critical period in the life of the family is in the phase of the initial diagnosis of the patient. In this period of inevitable shock, a series of reactions is mobilized and a restructuring of family relationships is brought about. This restructuring mobilizes adaptive and maladaptive processes and general reshaping of the relationships within the family.

Of course, these processes continue during the course of the child's development and the cycle of the life of the family. Whether, in the course of the development of the child and of the cycle of the life of the family, adaptive or maladaptive processes will finally prevail depends upon many factors. Some of these are the maturity of the parents, their previous experience of life, and the quality of their relationship. Another factor is the seriousness and complications of the illness and the occurrence of other destructive events for the child and the family, resulting in old traumas and conflicts coming to the surface and upsetting the existing equilibrium. At this point it must be stressed that it is important while working with these families to look for protective factors and coping mechanisms, in order to strengthen them, and at the same time to identify risk factors or maladaptive coping mechanisms, in order to diminish or eliminate them. It should also be said that a number of these families have succeeded—through the processes of chronic stress because of the illness—in developing satisfactory coping skills and successful strategies in order to deal with the effects on the child and the family, thus facilitating the normal psychosocial development of the children and maintaining normal family life as far as possible.

The endeavor to emphasize in recent years the study of the psychosocial effects of thalassemia on the patients and their families needs to be systematized. It will be recalled that in recent years a European Interstate Group under the aegis of the World Health Organization (WHO) Regional Office for Europe, Maternal and Child Health Division, has been formed, with support from the concerted action programs of the European Economic Community (EEC). This group is developing activities related to the improvement and evaluation of the effectiveness of the services for hemoglobinopathies. In collaboration with this group and on the initiative of certain specialists from the mental health professions, another group which is also under the aegis of WHO has been set up with the participation of centers from Britain, Belgium, Bulgaria, France, Italy, Greece and Cyprus, while centers from other countries (Romania, Brazil) have shown interest in taking part.

This latter group exists in order to promote research regarding recognition and treatment of psychological factors in thalassemia and sickle cell disease. Another aim is to define good practice and thus develop guidelines for the psychological management of such patients, with the ultimate aim of their optimal integration into society.[16] The group has planned a research protocol (FIG. 2) with an interstate study as its objective in the first phase. More than one center from a single country can take part in this study.

It is also considered necessary that the effort be continued to incorporate principles of psychiatry or child psychiatric treatment into the work of the medical and nursing staff, for a better psychosocial treatment of these families affected by a hemoglobinopathy. It is also important that the medical services should be backed up by interdisciplinary psychiatry or child psychiatry groups.

Also significant is the role which can be played by parents' and patients' associations in various countries; an example is the Thalassemia International

Federation for the improvement of the conditions of treatment and social care of the patients and their families, particularly in countries with scarce resources.

I also believe that, in the countries where the problem of thalassemia exists, it is necessary—at the level of a social policy and of a policy on health—for programs to be developed, together with services for prevention, the ensuring of a sufficient supply of blood, the giving of despheral and, generally, full insurance cover and management of the additional needs of people with thalassemia and their families.

Furthermore, there can be no doubt that it is necessary that action should be

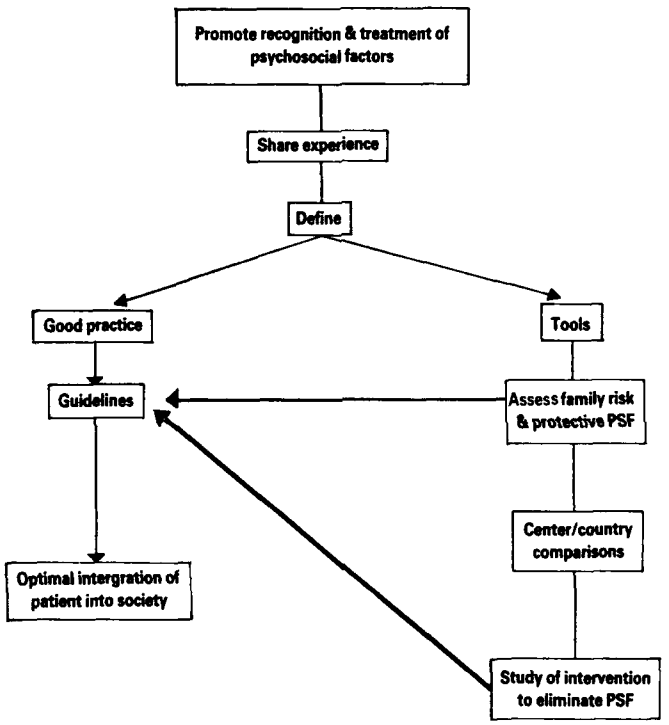

FIGURE 2. WHO protocol for research on the recognition and treatment of psychological factors in thalassemia and sickle cell disease and for development of guidelines for the psychological management of patients with hemoglobinopathies. PSF, psychosocial factors. (From Ref. 16. Reprinted with permission.)

taken at the individual, social, and state level with a view to the social integration of children with thalassemia, taking into account the particular features of the condition and the difficulties encountered by these children in the course of their development. But let us overcome these difficulties, remembering the words of St. Augustine: "Give as many instructions as are necessary, as much freedom as is possible, but, above all, boundless understanding and love."

Finally, of course, the aim of all the efforts which I have mentioned is that people with thalassemia should live happy and creative lives.

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Pediatric Hematologists and Adult Thalassemics

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INTRODUCTION

Until not long ago thalassemia major was considered a fatal disease, and patients did not usually attain adult age; but the prognosis has improved appreciably in recent years for cases treated adequately from early in life. A study performed recently in Italy[1] demonstrated that 94.4% of thalassemics born between 1970 and 1974 reached their 15th year. Now most children with thalassemia, if appropriately treated, have the possibility of reaching adulthood.

When the advances in bone marrow transplantation[2] and molecular biology are considered, it is evident that the hope of cure is no longer an illusion; and health care workers and the families of patients are increasingly optimistic. These new possibilities have made a re-evaluation of the entire intervention strategy necessary. Therapy must be planned with the long-term objective of ensuring normal development from childhood to adult age and social integration at the highest possible level. This problem has recently been considered by the World Health Organization (WHO) Working Group on the Psychosocial Aspects of Thalassemia and Sickle Cell Disease, which has prepared a leaflet entitled *Meeting the Needs of People with Haemoglobin Disorders: Recommendations for Psychological/Social Management and Support.*[a] Three particularly significant observations are made in this document, as follows: (*a*) "Psychosocial support is an integral part of the total management of people with hemoglobin disorders." (*b*) "Management of hemoglobin disorders requires the participation of a multidisciplinary team (the treating physician, working with consulting specialists, a psychiatrist and/or psychologist, nursing staff, social worker and other colleagues." (*c*) "The responsibility for overall management must remain with a single treating physician [the pediatric hematologist during the pediatric years]."

In fact, in recent years interest in psychological and social problems has progressively increased.[3] An evaluation of the social integration of thalassemics followed at two centers (Athens and Ferrara)[4] with long experience in overall care for thalassemic patients demonstrated, in two different areas of Europe with a very similar therapeutic standard, that it is possible to reach a good level of social integration. However, there are many problems to be considered, and prevented, starting in the first years of life. They include overprotection (in the family in particular), lack of self-sufficiency and independence, inadequate educational development, inadequate preparation for work, social marginality, and self-exclusion.

[a]This document is available from the Information Centre for Thalassaemia Associations, Clinica Pediatrica, Università di Milano, Ospedale San Gerardo dei Tintori, Via Donizetti 106, 20052 Monza MI, Italy.

Whereas it has been established that it is important to provide a therapeutic strategy that includes not only medical but also psychological and social interventions from childhood through to adulthood, the modalities and instruments of intervention and the methods of evaluation and verification still have to be defined. Moreover, the various organizational, cultural, and social situations must also be taken into account. In this complex strategy the pediatric hematologist has a central role; as the person responsible for the therapy he must be able to coordinate the various members of a multidisciplinary team and be prepared to consider not only clinical aspects of the disease but also educational, psychological, and social ones. No well-defined model exists, and it is necessary to find the optimal solution in accordance with the work setting, which can vary greatly in relation to the health care workers available, and the multiple environmental and sociocultural conditions.

Several years ago we started to implement an intervention strategy of psychosocial support for the child with thalassemia and his/her family at our center. We now present a brief report of our experience with reference to the organizational modalities and the instruments used, with particular regard to the pediatric hematologist's role.

OUR EXPERIENCE

Thirty thalassemic patients, aged between nine months and 20 years, are followed at our center by a multidisciplinary team that is coordinated by two pediatric hematologists (who spend about 50% of their time on this activity) and includes a social worker and a psychologist (for 20% of their time) and a nurse (50% of his/her time). A more complete description, including the rationale of the single interventions, has already been published.[5] The role of the pediatric hematologist in this strategy consists of the interventions described in the following paragraphs.

The Family

The initial approach to the family is a semistructured interview with the Spinetta questionnaire, which was based on experiences with families of patients with chronic diseases.[6] The interview, conducted by the pediatric hematologist and evaluated also by the psychologist and social worker, serves as a basis to define any problems and to program intervention modalities. However, only rarely (in less than 20% of the cases) are specific problems identified and specific interventions necessary. Subsequently, a formal meeting is scheduled at the time of each transfusion, usually short (about 10 minutes), to re-evaluate or define any problems. In addition, updating meetings and discussions of medical topics and care are also promoted. Lastly, the pediatric hematologist encourages activities of the Parents' Association.

The Patient

The aim in approaching the patient is to promote understanding of the disease and thus self-management by (*a*) group meetings with the multidisciplinary team each time the need arises; (*b*) a course of biennial lessons on blood and biological aspects, adapted according to age group and given by the pediatric hematologist, and

(*c*) study-holidays lasting some days, at periodic intervals, for children over 11, with the participation of the medical and psychosocial staff.

The School

Together with the social worker, the pediatric hematologist carries out a program of collaboration with the teachers and thus establishes a direct contact between the school and the center. One aim is to confirm that thalassemics can participate in normal scholastic activities and remove any prejudices and preconceived ideas. In this regard a booklet is distributed to the teachers that describes the characteristics of the disease and shows the importance of following a regular scholastic curriculum.

An annual check is performed of the cognitive, social, and emotional functioning of children with thalassemia in school. For this purpose we administer the Deasy-Spinetta Behavioral Questionnaire (DSBQ), which is already in use in the United States and Italy for children with leukemia.[7] Both the teacher and the parents complete the questionnaire, and the child's functioning in school is thus monitored so that problems can be recognized in time for early intervention.

Work and Sports

In the last year two study programs have been set up to provide vocational guidance and to promote sporting activity. The fundamental aim of the vocational guidance program is to define the aspirations and capabilities of each individual and to encourage vocational training from an early age. Sensitization of the family is also important to make them recognize the possibility of future employment for the child. In the sporting activity program, performed in collaboration with physiologists of Milan University, the possibilities and limits of each individual are evaluated. Our experience is still limited as regards these two aspects, but both programs have been received with marked interest and almost all the patients are active participants.

Adult Patients

There is a group of eight adult thalassemics who are followed at the Adult Hematology Center at our hospital, and this center applies the same intervention strategy as the pediatric team and collaborates with them. Activities are coordinated, with periodic meetings to discuss organizational matters and review clinical problems.

Although the pediatric hematologist has an obvious fundamental role in the care of children with thalassemia, whether he has a direct role in the treatment of adult thalassemics is not well defined. The situation in this regard varies greatly in different places, and thalassemics are not infrequently treated in pediatric centers regardless of their age. Considering that the number of adult thalassemics is continuously rising and that they present particular problems, we believe that suitable facilities should be available for them in departments of hematology or medicine. Physicians caring for adult thalassemics should consider all the experience gained by pediatric hematologists over the course of many years so that, with modifications appropriate to the difference in age, an intervention strategy may be implemented that is aimed at optimal quality of life and social integration.

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The Impact of Thalassemia on Body Image, Self-Image, and Self-Esteem

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How much do we value ourselves, how much do we really like ourselves? Have we really come to terms with who we are, how we look, what we have? Issues of self-esteem and self-worth are not only central to our psychological well-being but also determinant for how much we care for, and pay attention to, ourselves and our bodies. How much we like and value ourselves is closely related to how we view our bodies. We all have misgivings about our bodies. Some of them are realistic and a lot of them are unrealistic. For many individuals, having a crooked nose or small eyes, or being a little too short or a little too fat can be devastating to their self-image. Our consumer society places a heavy emphasis on body image for one's success as an individual. Body image is closely related to self-image. Then how should a chronic illness like thalassemia affect body image and self-esteem?

One of the most potent sources of stress is physical illness. When we are ill, even when we have the flu, our body suffers; we suffer. We cannot do all the things that we used to do; and, more importantly, we do not *feel* like doing too many things. When our body is ill, it causes us pain and frustration; it is, the source of anxiety and suffering. The question is always, When will I be well again? For the individual with a chronic illness, however, the answer is very bleak: You will never be well. You will always carry this illness with you. The illness is part of you. This is a very difficult idea to accept. It is not easy to incorporate the presence of the illness in your self-image and feel good about yourself. To the ears of the novice it may sound like a contradiction. Is it possible? Can it be that your body causes you pain and frustration and yet you like it and you take care of it?

The question becomes even more complicated when we think not only of the realistic hardships that a chronic physical illness presents to the individual, but of how this individual is treated and viewed by others. For many years the presence of thalassemia was identified with, and often still is considered as, a "stigma," a source of inferiority. The affected individual is not seen as a person with a physical problem but as a weak, incapable being for whom we feel sorry and to whom we offer our sympathy. Thus, what is incorporated in one's self-image is not only the physical illness but also a specific view and a specific attitude towards one's self. The internalized image of an inferior individual. Is this inferiority a realistic one? Does it mean that an individual with a physical illness is a weak and incapable being?

A review of the literature informs us that quite a few changes occur due to the presence of a chronic illness. The diagnosis of the disease is a devastating, heartbreaking, and earthshaking experience for the parents.[1-4] Very often parents react with intense grief; they feel sad, depressed, guilty, and disappointed. The diagnosis also

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brings changes in the parents' relationship with the child. They may become critical and rejective or they may become overindulgent and overprotective, limiting the child's already restricted functioning. Very often parents become lax with their discipline, allowing the child to have the most important position in the family. The child functions as the center of the family unit, often interfering with the mother-father relationship. The family may face further problems because the child is very close to the mother, who just takes care of the ill child and neglects her husband and the other children in the family. Siblings may become jealous of the ill child. As a result of these feelings of being neglected and left out, intense rivalry and/or psychological problems may develop in the other children, in an attempt to attract the attention of the parents.[5-8]

These faulty patterns of functioning and communication within the family may be extended beyond the nuclear family to the relationship of the parents with their relatives and friends. Often the parents are secretive; they feel ashamed of having an ill child and do not discuss it. Feelings, thoughts, worries, fears, anxieties, etc., are not discussed but are kept inside each individual, leading to further problems.[1,9] The ill child is isolated from peers and is hidden from society. Very often, in the not-so-distant past, children with thalassemia were not allowed to go to school; they were not allowed to play or to be involved in all the normal activities in which a young child should be involved so that he/she can develop an adequate self-image and self-esteem.[10-12]

Following the example of the parents, children also become secretive and do not externalize how they feel. They do not talk to friends about the illness, fearing that they will be rejected and treated differently. Thus, they often do not get involved in all the normal activities of their age. They often feel that there is something wrong with them, that they are inferior, inadequate, less than or worse than others. These problems of self-esteem are often compounded by physical differences which lead to a poor self-image and to feelings of depression and hopelessness.

Is this bleak image that is presented to us reversible? Can it be that an individual with thalassemia, or with any chronic illness, feels good about himself/herself? Can it be that an individual with a chronic illness adjusts and successfully copes with this difficult problem? I will present two case studies which will help us to address these questions.

Julia is a 28-year-old woman with thalassemia. She has been in therapy for two years. When she first entered therapy, she related that the most important issue for her was her relationship with her body. "I don't have a good relationship with my body. I don't like it." She stated her goal as follows: "I want to feel stronger; I want to feel that I can shape situations without them shaping me. . . . When I meet with difficulties I quit. . . . I get easily disappointed."

During the first year of therapy Julia discussed primarily her difficulty in expressing herself, in expressing how she felt and what she thought because of her fear of being rejected and criticized. In addition, she always felt guilty when she tried to defend herself and what she wanted and needed, as if she did not deserve to have what she wanted. Her view of herself was that of a sick individual who was unwanted and unattractive. She felt that her own family devalued her and did not believe in her. She was very angry about it but never expressed this feeling openly.

Suffering from a low self-esteem and a devalued sense of self, Julia always looked for approval and acceptance from others. In her relationship with men she also wanted approval and affirmation of her femininity. However, she felt very easily hurt and rejected, and she believed that the only thing she could offer to keep a man was sex. Yet, she did not enjoy her sexual relationships because she did not feel

comfortable with her body. Furthermore, she believed that her partner would not like her body and general appearance, although she had no physical deformities.

When discussing her feelings of weakness and vulnerability, Julia related that the overprotection of her mother made her feel weak and fragile. Why would her mother otherwise be so worried about her? She always received the message that she must be very careful with everything she did because it would be very easy for her to "break." She was able to realize that this was what she now thought of herself, how she came to view herself. Every time she would take a risk, either physical or emotional, she would feel she was in danger of breaking down, of hurting herself.

Julia's fear of being hurt, of causing damage to herself, of dying, was a very important issue that prevented her from enjoying life. She experienced great difficulty in expressing herself and her fears and used to somatize all of her problems. Whenever she did not feel well and wanted to cry, she would hold it back and would instead talk about a terrible pressure she had in her chest, how her stomach felt bloated, how much her spleen hurt her. Julia related that when she was growing up, her illness was the only thing that her parents, and other adults around her, seemed to take seriously; it was only with somatic complaints that she could express herself and draw the attention of others. In contrast, the expression of emotions was something that was prohibited. The family never spoke openly about how they felt in general or about the illness.

Part of the therapy focused not only on her relationship with her parents—who viewed her as fragile, different from the rest of the family, and in need of their constant support and caring—but also on her relationship with doctors and nurses. They also seemed to reinforce this view of her as fragile and in need of special treatment. However, what was even more painful was that they did not seem to "see her." They just saw her body and its vulnerabilities. They took care of her body but never asked her how she felt. Julia realized that she had always been afraid that others would not "see her," would not care about how she felt; and, even more, she realized that she believed she should not say how she felt.

As she slowly became more able to express her pain and sorrow about her condition, her feelings of weakness, fragility and inferiority, her somatic symptoms were reduced; but she was then hunted by nightmares. Nightmares of falling, of hurting herself, of dying, of being alone. Recently Julia had a dream. She dreamt she was naked and her body was the object of observation. She felt very ashamed of her existence. She related that she realized how much she hated her body because it made her suffer, how much she hated her existence, and how afraid she was that she would be self-destructive and hurt herself. She was experiencing a lot of sorrow and despair. It was, however, the first time that she could express so openly how terrible she felt and was able to cry in the presence of another human being. Since then Julia has been expressing more and more her concerns, her anxieties, her fears, her difficulty in reconciling herself with her body. She has been able to be more open and expressive with her friends and especially with her boyfriend. She complains less of physical issues and has started smiling more often. She speaks with a voice that is more self-assured and is seriously thinking of starting a small business.

Iphigenia is a 30-year-old woman with thalassemia. She has been in therapy for three years. When she first entered therapy, she wanted to learn more about herself and her relationship with others, primarily men. She related she had an image of a big castle which she was about to enter. She was very scared of what she would meet inside: monsters and huge spiders and all sorts of scary things.

Iphigenia, like Julia, had great difficulty in expressing her feelings and her concerns. It was very difficult for her to express her anger and her resentment for her condition, because she was afraid that others would reject her. She had learned that

she had to be brave and strong in order for others to approve of her, and she never wanted to show that inside she felt weak and vulnerable. She needed to be in control and could not relax, because if she did, she was afraid she was going to be self-destructive and would hurt herself: that if she let go, she would slowly but surely kill herself by not doing what she had to do, by neglecting her medical treatment.

As therapy proceeded, Iphigenia was more able to express her feelings of weakness and vulnerability, her deep concerns about dying and about not having enough time to do what she wanted to do. She became very angry with others and resented the fact that she had to suffer so much whereas they did not. She resented the fact that she never lived a carefree life. Even as a child she always had to be aware of when she had to go for a transfusion, had to remind herself to use her desferal, had to make appointments with doctors. Her life was full of hurdles that she had to overcome, and she often felt very tired of taking care of herself. During such times she would get depressed, would not use her pump, and would just hang out with friends, endlessly talking about whether there was any meaning to life.

Iphignia related that one of her greatest fears in expressing herself and her feelings to others was that they would not take her seriously. She felt very deeply hurt when she opened up and then felt that she was not understood, that others did not *care* to understand. As she proceeded to explore this issue, Iphigenia realized that one of her most important traumas came from her relationship with doctors and nurses. She remembered how she felt as the object of observation and how hurt she was that they just looked at her body. They did not "see her." They just saw her body. They did not care to see her, but only cared about the body, this body that she hated so much because it was the source of all her misery.

It was not until much later in therapy that Iphigenia was able to reconcile herself with the idea that she did not like her body but it was still the only one she had. She did not like what she had and what she needed to do in order to keep fit, yet she liked herself. She believed that her illness gave her a wisdom that was far beyond her age. She was able to realize that life is valuable and that life is what we make it to be. She did not want to waste any of her precious time. She wanted to be productive and to live as fully as she could. Iphigenia is now a successful woman, admired by her colleagues for her liveliness, her endurance, and her ability to solve problems and deal with frustrating situations that incapacitated them.

Julia and Iphigenia are two examples that can help us understand a number of important issues, as discussed in the following paragraphs.

How the illness is perceived and dealt with by others—parents, doctors and other significant adults—may cause more of a problem than the illness itself. It is very important to understand that the child is greatly influenced by how adults view him/her. Our self-image and self-esteem is to a large extent dependent on, and affected by, the perceptions of others. When your own mother and father view you as a weak and vulnerable individual, it is more than likely that this is how you will also think of yourself.

When parents overprotect or do not discipline their child, when they do not allow it to do all the things that a healthy child would do, they indirectly say to the child: You are vulnerable, you are fragile, you are different from the rest of the kids. In the mind of the child, this difference is translated into the idea that he/she is less than the other children. Parents have to be supported and taught how not to transfer to their child fears and anxieties that they experience. Some of these fears and worries may be realistic; a lot of them, however, have to do not with the actual condition of the child, but with our misconceptions of how terrible it must be to be ill, with our own fear of death and vulnerability.

In this respect, doctors and other health care providers could be of immense help.

Yet, they very often make things worse, since they are themselves not well informed or are thinking too much of the child's physical well-being, neglecting to think of his/her emotional well-being. It is a common complaint that doctors "give lectures and tell us what to do but they do not know everything." This everything has to do with the child's psychological state. It is easy to say that you must use your pump every day, but it is very difficult to empathize with what that means for the individual. What a struggle it is to constantly do what you *have* to do and *do not want* to do. Obtaining compliance with medical treatment is something relatively easy when the child is young and the parents cooperate. It is very difficult, however, when we deal with teenagers, who in any case do not want to comply with anything; and with adults, who do it because they *have to*—although they do not care—and not because they want to live. The question then arises: can we help someone to *want to live?*

How and whether feelings, worries, and concerns about the illness are expressed is of immense importance. Life without emotions is like food without spice. When we are unable to experience our feelings and express them, it is doubtful that our life has much meaning. It is important for all people to be able to express themselves, to say how they feel and what they think. Very often, however, we do not express ourselves, because we are afraid. One of the most common fears is that we will be rejected or not liked by others. The individual with thalassemia often faces an added complication. Discussing feelings and thoughts about the illness is taboo. We all know but no one speaks. This secrecy is a very common phenomenon in other taboo areas like incest or child abuse. The message is that this is something "bad," which must not be discussed with others and/or the family. It is also important to remember that in the case of abuse and incest the blame is often placed on the victim and is internalized: "If I had been good enough dad would not have beaten me." "If I had not been provocative I would not have been harassed." One has to wonder whether the question is not raised for the ill child too: "If I had not been bad I would not have been punished." Having to go to the hospital for these painful procedures—what else could it be than a punishment for unknown sins? Sins with which often the parents themselves feel that they are haunted and for which they are punished through the ill child.

Fear of death, anxiety, anger and depression are common and say nothing about the mental health of the individual. George Vaillant in his book *Adaptation to Life*[13] wrote:

> The mentally healthy are by no means immune to anxiety and depression. This is because healthy adaptation requires an accurate perception of the universe, and accurate perception often evokes pain. . . . The reliable presence of people who love us facilitates our perception and toleration of painful reality and enriches our lives. . . . *Soundness is a way of reacting to problems not an absence of them* [italics added].

The presence of a chronic hereditary illness like thalassemia is a very demanding condition that will unavoidably cause a number of intense emotional reactions. The accurate perception of the implications of such an illness cannot but create feelings of anger and depression, fears and worries. If the individual does not experience them, he/she is not in touch with reality or is refusing to deal with the reality of his/her condition, in which case we would talk about a maladjusted person. The issue then is how one deals with these emotions. A first important step is that we allow the individual to have them, to experience them and to express them.

It is the parents, the doctors, the nurses, and all who care for the well-being of the individual who have not only to look at the body but also to *care* about how this person *feels*. From research with cancer patients who are dying we know that it is not the dying person who does not want to talk about his/her imminent death, but it is we

who "care" about them who cannot tolerate to talk about it, who cannot tolerate our fear of death.[14] The same applies with chronically ill individuals. They know that they are ill and are dealing with it, since they anyway cannot do otherwise.They need to talk about how they feel; but they have not learned how to do that, since everyone has always been avoiding such discussions. We usually avoid discussing something that we are afraid of, something that touches on our fears. Understanding that these feelings are normal and that it is all right to express them greatly reduces their impetus. Whatever is left unspoken, half hidden, or unconscious acquires a tremendous power over us and directs us.

The presence of a chronic illness can be growth promoting if it is not viewed as "God's verdict" for a miserable existence. No one can deny that the presence of a chronic hereditary disease like thalassemia is a serious problem and a serious crisis that confronts the individual and his/her family from very early on. However, we must remember that for the Chinese the word crisis is written by two symbols: *danger* and *opportunity.* There is a threat that has to be dealt with but also an opportunity for growth. As George Vaillant[13] stated:

> Serious physical illness may contribute to mastery of life. Thus, inner processes can erase or magnify the effects of external illness. Health and the "ego" must be considered together. . . . An outstanding feature of successful adaptation is that it leaves the way open for future growth.

The question then is raised: How can we successfully adapt to the presence of thalassemia? Taking into consideration all that has been mentioned before, we could say that the single most important factor is our *attitude* towards what we have: how the illness is viewed and how it is incorporated into one's self-image. Is it that we *are* ill or is it that we *have* an illness? In other words, is it that we are ill and thus weak, fragile, and all of the rest of our misconceptions or is it that we have an illness, which implies that we are in control of the situation? Very often the presence of the illness overpowers all other aspects of the individual's functioning. He/she *becomes* the illness. However, it is always important to remember that we are what we think we are. When we equate our existence and our self-image with the illness we have, we say that this illness is the most important thing, we give it the power to control us.

It is the responsibility of all those who are involved with thalassemia, and of course individuals who have thalassemia themselves, to realize that the illness is not the most important aspect of their life. Life is much more than an illness, and when one is able to deal with such a difficult problem one is able to deal with all of life's problems. When we are able to face the unavoidable reality that we will die, when we come to terms with our existence, we can start to *live a meaningful life.*

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A Patient's Perspective

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My name is Ralph Cazzetta. I am a patient with Cooley's anemia, and I am president of the Thalassemia Action Group, a patient self-help group sponsored by the Cooley's Anemia Foundation.

I am 24 years old, a high school graduate as well as a certified interior designer, and am currently employed as an office manager for a heating and air conditioning firm. My twin sister is a certified social worker, and both my parents are professionals. My older brother, who by now would have been 31 years old, died of Cooley's anemia when I was 5 years old. I recall that night often, as well as the many days when we were transfused together. These things I wish to both remember and forget.

My transfusion regimen requires two units of blood every two weeks to maintain a hemoglobin of no less than 10 so that I can try to function as normally as possible. My desferal treatment, which includes the use of the infusion pump as well as bimonthly intravenous administration, is costly, painful, and a psychosocial intrusion in my life, as well as the lives of my family members.

I am hopeful this brief biographical sketch, which describes a life that I believe is comparable to that of many Cooley's anemia patients, will give you a deeper and greater awareness of the many physical and emotional problems and decisions that we who live with Cooley's anemia must face every day of our lives.

Before presenting a sketch of my life, I would like to tell you a little about the history and purpose of the Thalassemia Action Group (TAG). TAG is a patient support group (FIG. 1); at one time, such groups were non-existent. It was only recently, in the summer of 1985, that TAG was formed and patients had one another to turn to. Before TAG we were in the dark, but now we are in the light and can speak for ourselves about our disease.

Medical research has come a long way in the treatment of Cooley's anemia. However, with each new advancement, such as chelation or bone marrow transplantation, we patients and our families are forced to make decisions concerning the pros and cons of these new therapies and the effects that these advancements might have on our quality of life.

As we all know, transfusion therapy is currently the only means of survival for those afflicted with Cooley's anemia. And with such therapy comes concern for the quality and quantity of our blood supply. Because of the constant shortage of blood, will we be able to receive our transfusion? Will the blood be safe? Our biggest concern today is whether our blood supply is free of infectious diseases such as AIDS and the hepatitus virus.

We know that transfusions are the key to our survival. But, unfortunately, transfusion therapy poses to the patient a new threat—iron overload. This now becomes another medical problem, as well as another psychological matter for patients and parents.

The problem of iron overload mobilized researchers to produce another regimen

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THALASSEMIA
ACTION
GROUP

Uniting for
SUPPORT
KNOWLEDGE
UNDERSTANDING

What is TAG?

TAG, Thalassemia Action Group, is a network of thalassemia patients 13 years old and over who have joined together to develop support groups around the country.

Together we have developed specially designed programs to help fulfill our needs.

Purpose

...Reach out to all thalassemia patients to offer love, support and a learning experience.

...Provide young adult patients a channel of communication for support and to share life's 'ups and downs.'

...Promote compliancy with the treatment of desferal using the "pump."

...Encourage a positive attitude toward life.

Activities

...Conduct a national annual TAG Conference promoting understanding and knowledge.

...Conduct regional meetings offering support, friendship and knowledge.

...Maintain a network of young adult patients serving as regional coordinators to offer support in any situation.

...Award yearly scholarships to thalassemia patients.

...Publish a bimonthly TAG newsletter to keep patients informed of current activities and advancements in treatment.

...Testify before congressional committees for research, education and counseling funds.

...Assist patients with insurance problems.

...Fight against employment discrimination.

...Improve relations with local hospital facilities.

...Help create transfusion centers.

...Provide counseling.

FIGURE 1. Thalassemia Action Group (TAG). This excerpt from the TAG brochure describes the goals and activities of the group. (Reprinted with permission of the Thalassemia Action Group.)

of treatment, chelation therapy, which is equally as important as transfusion therapy. Desferal, an iron chelator, was introduced in the early 1970s. Although chelation therapy has proven able to extend our lives, it has also proven to be the most difficult treatment for the patient to endure. Not only is chelation therapy painful to administer, it also requires nightly infusion for a period of 12 hours. For many, this is not only painful, but at times impractical—and it certainly curtails one's social life. We and our families are now responsible for preparing and administering the drug. Because of the 12-hour requirement, most of us find this to be a difficult routine to integrate into our daily lives; thus, compliancy becomes a major issue.

Recently, bone marrow transplantation became a real possibility as a cure. For those who meet the requirements and are good candidates, this procedure can afford a life free from transfusion and chelation therapies. One must remember that although a bone marrow transplant is an alternative to these therapies, choosing to undergo it remains a personal decision. However, once a decision is made, those involved are faced with many fears and concerns, such as the possibility of tissue rejection and of death. Many questions still remain regarding the transplants and the

survival rate of patients. In the future, I'm sure gene therapy will also present the same dilemmas.

As our life expectancy is extended, we now share the same hopes and fears as those not afflicted with this disease. In the past, our short life expectancy meant that higher education, long-term career goals, and establishment of relationships and marriages could not be considered. But now, some of us have found it possible to achieve our dreams of becoming teacher, dentists, lawyers, and, yes, even medical doctors.

Ten years ago, no one thought that a patient would ever survive long enough to reach his or her life's dreams. Many of us never dreamed that we could hold full-time jobs or become professionals. High school was the education limit of a thalassemia patient. But now, because of the ongoing research and treatment, we see many patients achieve life-long goals—but not without major problems and obstacles to overcome. It is not easy to go to school, to work, have friends, socialize, and live normally when we face bimonthly transfusions, nightly chelation, and fear of infectious diseases. And, in many instances, even our physical appearance becomes a hardship. However, in spite of all these conditions we are grateful for the additional years we have gained.

With the new treatment and therapy, the quality of our lives has improved. For example, relationships are now a real possibility for a patient. We have gained the confidence we once didn't have because fear of rejection was in the back of our minds: Will people turn away from us because of our disease? Will they fear our deaths?

However, it appears that each time we overcome one obstacle, we face another. The issue of rejection by people has taken a new and stronger form. Today some patients have the burden of dealing with the fact that they have tested positive for the HIV virus. They now have to not only explain to their partners or spouses about thalassemia, but also talk about the AIDS virus. They have to discuss the risks and the effects on the future of their relationship. So we now must take into account a future or present spouse and how infections will affect a relationship, a marriage, or a friendship. Yes, advances have been made. Yes, we are living longer, and of course no one wants to be negative or depressing. But the fact remains that these issues affect our everyday living.

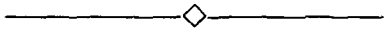
We are living longer, getting proper education, holding jobs, and even entering professional fields—all the makings of a normal life. We are told by health professionals that we should become who we are, plan our future, and live as normally as possible. We agree; it sounds wonderful, but the reality is that it doesn't always work that way. We make it through school; we go for jobs and then must face applications or interviews which ask about health. We must then decide whether to tell the truth or not. If we're hired, will they give us a day off every other week for a transfusion? Will they tolerate our absences because the hospital does not have evening or Saturday hours? For the younger patient, how many days of school are missed? For the college or graduate student, how much work and time must be made up?

Yes, the therapy has improved; we are living longer. But the problems become more complex and the solutions fewer. This is an area that the health professional team needs to address. At whatever point we are in our lives, whether it's in elementary school or college, or working at a job; whether we are married, single, dependent or independent, these are the "realities" of life and they are just as important to us as to those who are normal. Yes, we would like to experience being normal. We would like to see schools understand our problems and yet provide us with a full education. We would like to tell employers that we have special needs and have them understand. We would like to see hospitals and doctors understand that

the older patients cannot meet their nine-to-five schedule. We all need to work together—patient, teacher, employer, doctor, hospital, and all those who can help us improve our quality of life. We must remember that quantity of life does not guarantee quality of life. Both require care, cooperation, and understanding.

As a patient of the nineties, I look forward to new therapies and a cure for all thalassemics around the world. But as we wait, I look forward to a more active role between patient and school, community, doctors, and hospitals for a better and more fruitful life. *We must treat the spirit as well as the body.*

Thank you for your kind attention.



I would like to dedicate this presentation to the memory of my very best friend, Thomas Mirabella, also a Cooley's anemia patient, who passed away two years ago. He and I once talked about how one of my goals was to speak before medical doctors about what the patient goes through; and I recall Tommy telling me to be patient, that the time will come—and it has.

Closing Remarks

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It is a pleasure for me to make a few remarks to close the conference. I believe this conference marks a milestone. It is the first conference in which we have focused our attention on accurate diagnosis and the hope for new therapies to cure the disease. We heard about extraordinary, new and simple ways to diagnose and detect Cooley's anemia; and we heard about a variety of new iron chelators, about bone marrow transplantation, fetal hemoglobin synthesis, and the possibility of gene therapy—all focussed on curing the disease.

On the first day of the conference, we heard from Dr. Haig Kazazian about the defects in Cooley's anemia and from Dr. Douglas Higgs about α-thalassemia. We believe we now have identified most of the genetic defects in these patients and know how they cause disease. Dr. Y. W. Kan then introduced us to the new and vastly simplified technology for detecting these defects, the polymerase chain reaction or PCR, which allows us to amplify the globin genes a million or more times in a few hours. This provides an incredible tool for use in identifying any defect quickly and accurately.

We heard about the great progess being made in defining the particular defects present in different populations of patients with thalassemia—in Thailand, Algeria, Israel, Italy, China, Turkey, Sardinia, and Greece.

We then closed the first day with an extensive discussion of how we might use increased fetal hemoglobin levels to cure Cooley's anemia. Advances in our understanding of the regulation of the expression of fetal and adult human hemoglobin in general have been extraordinary over the past several years. In particular, Dr. Frank Grosveld's observations of DNA sequences that can vastly increase fetal and normal adult hemoglobin may soon be applicable to the treatment of patients with this disease.

On the second day of the conference, we heard about the extraordinary advances being made in preventing Cooley's anemia by antenatal diagnosis. Antenatal diagnosis now is so simple and convenient that it can be carried out throughout the world. The work of Drs. Stuart Orkin, Kazazian, and Kan has paved the way for this technology. But it is Drs. Antonio Cao in Sardinia, Dimitris Loukopoulos in Greece, Jizeng Zhang in China, and others in countries where the disease is prevalent, who are the real heroes in applying this work to prevent the disease. In Greece and Sardinia, 80–90% of Cooley's anemia has been eradicated in the past five years by antenatal diagnosis. This in my mind is the crowning achievement to date of our work on this disease.

In the afternoon, we talked about the current therapies for thalassemia and the potential use of oral iron chelators. We have a useful and improved current therapy of blood transfusions and iron chelators, which is prolonging the lives of patients.

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However, we want a cure, and oral iron chelators offer that possibility. An extensive and heated discussion about the appropriate use of oral iron chelators was directed by Dr. Sergio Piomelli. It seems to me we will learn a great deal more in the near future with better controlled clinical trials of these drugs.

On the third day, we heard about the future therapy of thalassemia. Drs. Richard O'Reilly[b] and Guido Lucarelli told us they can cure Cooley's anemia by bone marrow transplantation, and the results are quite exciting. Immunologic problems still limit the number of patients who can receive this therapy; the possibility of complications also is a problem. But the results are becoming better and better. As Dr. Lucarelli told us, for a young child with a compatible donor, this may be the treatment of choice.

We then heard about potential longer-term cures of Cooley's anemia using gene transfer and gene therapy. Progress in this area is proceeding at a rapid pace. The results presented by Drs. David Bodine and Arthur Nienhuis were of particular interest in showing that monkeys can be given new genes. Recent advances have made it clear that progress is being made to make human gene therapy more acceptable to the public and the biomedical community. If successful, gene therapy will rapidly become a treatment of choice and potentially a cure.

On the final afternoon, we addressed the important psychosocial aspects of thalassemia, that is, the problems of patients with this chronic disease. I was proud of this session because it marked the first time that a large number of patients, their families, and physicians shared their thoughts at a Cooley's Anemia Symposium. Drs. Eugenia Georganda and John Spinetta[b] stressed that we tell thalassemia patients that their attitudes must and should be to try as hard as possible to lead normal lives with the expectation that we will soon find a cure for thalassemia. All our results point in this optimistic direction. It is difficult to think about Cooley's anemia in the 1990s in any other way. *Life is much more than an illness.*

I think the interactions among physicians, other health care professionals, and patients are critically important. I especially want to thank Mr. Robert Ficarra of the Cooley's Anemia Foundation for all his help in optimizing these interactions at this conference. I think Mr. Ralph Cazzetta and TAG (Thalassemia Action Group) succeeded well in focusing our attention on the needs of thalassemia patients. Ralph, we are doing as much as we can to try to cure this disease.

I also think the round table discussions, the poster sessions, and the cocktail hours allowed many participants from different parts of the world to come to know each other and to share their ideas informally with each other. We truly had an international conference with many, many countries represented from all around the world.

In closing, I want to thank all the participants, as well as the audience, for contributing to this meeting. I think the forthcoming *Annal of the New York Academy of Sciences* based on this conference will be most interesting. I also want to thank the New York Academy of Sciences for all its help in arranging the meeting.

I hope that the next Cooley's Anemia Symposium will be able to have as its main topic of discussion a comparison of the cure rates for Cooley's anemia using different therapies. Until then, I wish you all well and a safe trip home.

[b]Presentation not included in this volume.

POSTER PAPERS

Mapping of Deletional Forms of α- and γδβ-Thalassemia

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A wide variety of deletional events has been described whose 5′ and/or 3′ breakpoints occur within the globin gene clusters.[1,2] Analysis of deletion breakpoints mapping outside the clusters may provide information about mechanisms of recombination, as well as identification of informative unique-sequence polymorphic markers, mini-satellite and hypervariable DNA regions which can be used in determination of linkage to genetic disorders which map to human chromosomes 11 and 16.[3]

We describe two large deletions encompassing the entire α- or β-like globin gene clusters in two unrelated families (FIG. 1). In TABLE 1 are data for various family members on values for hemoglobin (Hb) and mean corpuscular volume (MCV). The α-cluster deletion occurs in a family of northern European extraction, segregates in three generations, and is at least 110 kb in size. The 5′ breakpoint extends beyond the 5′ hypervariable region (HVR), mapping approximately 70 kb 5′ to the ζ gene, while the 3′ breakpoint extends beyond the 3′ HVR, mapping 8 kb 3′ to θ.[4] The β-cluster

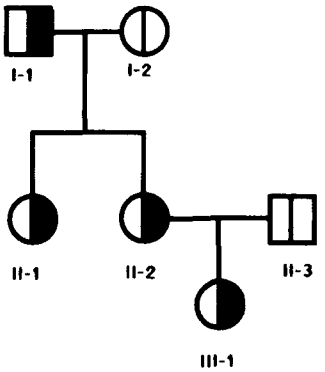


FIGURE 1A. Pedigree of family with α-cluster deletion. Affected members are indicated by *shading*. Hematologic data are shown in TABLE 1.

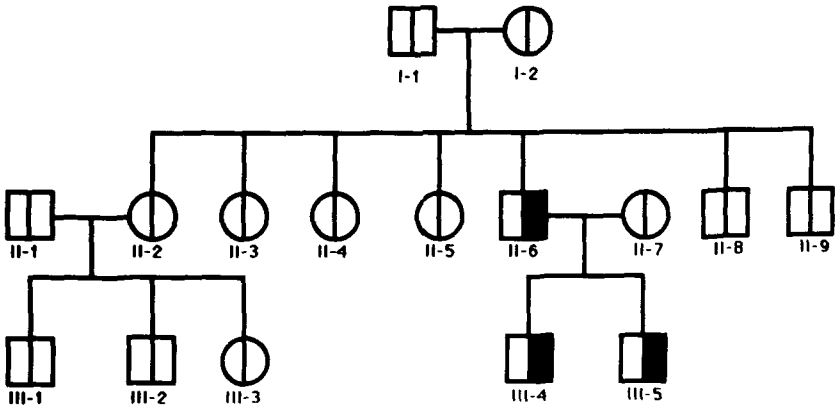


FIGURE 1B. Pedigree of family with β-cluster deletion. Affected members are indicated by *shading*. Hematologic data are shown in TABLE 1.

TABLE 1. Hemoglobin (Hb) and Mean Corpuscular Volume (MCV) Data for Various Family Members in Family with α-Cluster Deletion and Family with β-Cluster Deletion

Family Member[a]	Hb (g/dl)	MCV (fl)
α-Cluster deletion (α-thalassemia)		
I-1	12.4	71.5
I-2	14.7	90.9
II-1	10.7	64.4
II-2	11.7	67.4
II-3	15.4	96.6
III-1	10.5	64.0
β-Cluster deletion (γδβ-thalassemia)		
I-1	15.7	93.0
I-2	15.3	90.4
II-1	N.D.[b]	N.D.[b]
II-2	11.9	91.8
II-3	12.2	90.1
II-4	14.8	96.8
II-5	14.7	91.8
II-6	13.6	63.5
II-7	18.2	94.9
II-8	15.8	87.5
II-9	14.3	92.6
III-1	15.6	93.0
III-2	14.3	90.4
III-3	12.7	90.8
III-4	11.0	59.0
III-5	11.3	58.1

[a]Pedigrees for these two families are shown in FIGURE 1.
[b]N.D., not done.

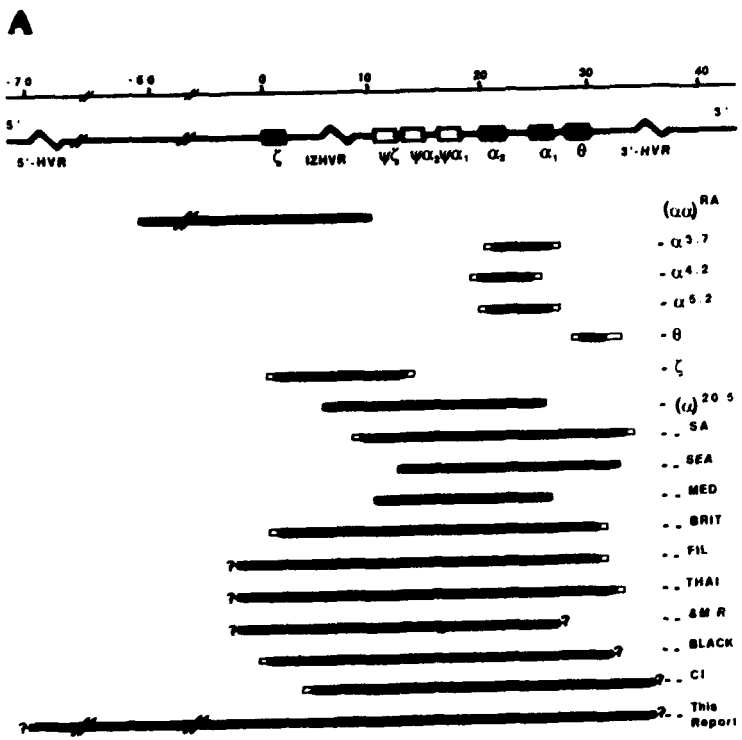
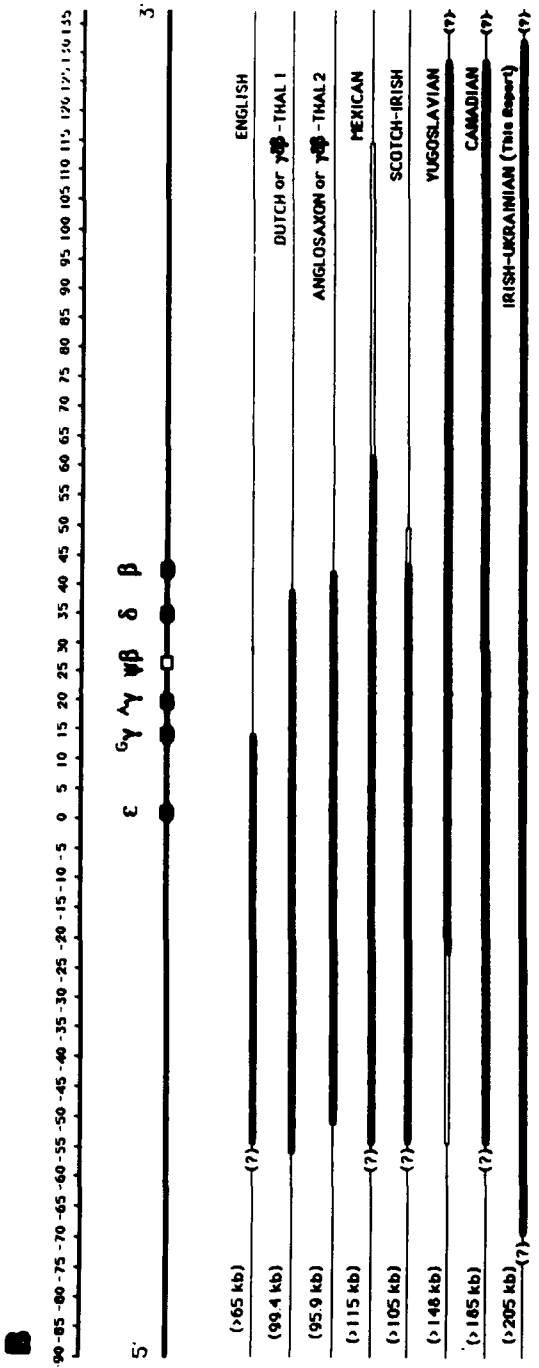


FIGURE 2. Summary of large deletions within and encompassing the globin gene clusters. Map of the (**A**) α-like and (**B**) β-like globin gene clusters, with positioning of deletions. *Unfilled bars* at ends of deletions represent possible locations of breakpoints; cases in which the position of the endpoint is not known are indicated (?). Names of various deletions are as previously described.[1,2]


B
-90 -85 -80 -75 -70 -65 -60 -55 -50 -45 -40 -35 -30 -25 -20 -15 -10 -5 0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90 95 100 105 110 115 120 125 130 135
5'
3'
ε
Gγ Aγ ψβ δ β
ENGLISH
(>65 kb)
(?)
DUTCH or γδβ-THAL 1
(99.4 kb)
ANGLOSAXON or γδβ-THAL 2
(95.9 kb)
MEXICAN
(>115 kb)
(?)
SCOTCH-IRISH
(>105 kb)
(?)
YUGOSLAVIAN
(>148 kb)
(?)
CANADIAN
(>185 kb)
(?)
IRISH-UKRAINIAN (This Report)
(>205 kb)
(?)

deletion occurs in a family of Irish-Ukrainian descent; it segregates in two generations, with affected family members having a β-thalassemia minor phenotype. The deletion is not present in the first generation, indicating a *de novo* event. The minimum size of this deletion is 205 kb; and it extends at least 15 kb upstream of the 5′ breakpoint of the Dutch γδβ-thalassemia deletion, which maps 55 kb 5′ to ε. In addition to removing the entire β cluster, this new deletion extends for at least 90 kb 3′ to β, mapping beyond the 3′-deletion breakpoints associated with hereditary persistence of fetal hemoglobin–1 (HPFH-1), HPFH-2, and Spanish $(\delta\beta)^0$-thalassemia.[5] A summary of the extent of the α- and β-cluster deletions is shown in FIGURE 2.

A variety of cosmid and yeast artificial chromosome (YAC) clones mapping outside the clusters is being used in combination with Southern blotting and contour-clamp homogeneous field electrophoresis in an effort to delineate deletion breakpoints. The cosmid probes D16S21 and D11S12, which map approximately 1.5 Mb from the α and β clusters, respectively, were present in two copies in all family members and unrelated controls, indicating the deletions do not extend into these regions. Current efforts include the use of probes generated by polymerase chain reaction (PCR) end-rescue of YAC clones[6] to perform chromosome walking studies from the normal α and β clusters. These end-rescued probes will facilitate analysis of gene dosage in normals and in the patients with the α- and β-cluster deletions.

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Sardinian Haplotype II β^0-Thalassemia Is Linked to the Variant $^A\gamma^T$-Globin Gene with a 4-Bp Promoter Deletion and Diminished $^A\gamma^T$ Expression[a,b]

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Several factors influence the severity of homozygous β-thalassemia: β-thalassemia intermedia (non-transfusion dependent) patients often have at least one β^+-thalassemia allele with only moderate impairment of β-globin production.[1] β-Thalassemia is also ameliorated by coinheritance of α-thalassemia or by γ-globin promoter mutations, such as T at position −158, which increase fetal hemoglobin production.[2]

In Sardinia, the codon 39 nonsense mutation (β39) is the primary cause of β^0-thalassemia.[3] Two-thirds of northern Sardinian patients have this mutation on haplotype II, while one-fourth have it on haplotype I.[4] Haplotype II, with ++ at the *Hin*d III sites in the $^G\gamma$ and $^A\gamma$ genes, is strongly associated with the variant $^A\gamma^T$ globin,[5] while haplotype I is associated with the normal $^A\gamma^I$.

We have previously demonstrated that a β^0-thalassemia haplotype found in a black family, with ++ at the *Hin*d III sites, had a 4–base-pair (bp) deletion at positions −225 to −222 of the $^A\gamma$ gene, in association with reduced $^A\gamma$ (elevated $^G\gamma$) levels.[6] We have now extended this study to northern Sardinia, providing statistical data for the association of the 4-bp deletion with decreased $^A\gamma^T$ expression.

To test the hypothesis that the 4-bp deletion is associated with haplotype II in Sardinia, 75 severely affected β-thalassemia homozygotes were haplotyped, and slot blots of polymerase chain reaction–(PCR) amplified $^A\gamma$ promoter DNA were hybridized with 19-mer oligonucleotides for the normal sequence (N), ATTAAGCAGCAG-TATCCTC, and the 4-bp deletion (M), ATTAGCAGTATCCTCTTGG.

Nine homozygotes for haplotype I all lacked the 4-bp deletion (i.e., they were N/N). All 26 haplotype I/II heterozygotes were N/M. Of 35 haplotype II homozygotes, 34 were M/M and one was M/N. One haplotype II/VI heterozygote was M/M (haplotype VI is like haplotype II in being ++ for the *Hin*d III sites in the $^A\gamma$ gene). Of the remaining cases, the three haplotype II/III heterozygotes were N/M, and the

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one haplotype I/III heterozygote was N/N. These data clearly show a strong association of the 4-bp deletion with haplotype II: for haplotype II homozygotes, only one haplotype II chromosome out of 70 lacked this deletion.

The relative expression of the $^{A}\gamma^{T}$-globin gene with the 4-bp promoter deletion was examined. The percentages of each γ chain in the fetal hemoglobin of β^{0}-thalassemia homozygotes were analyzed for six haplotype II homozygotes who were N/N, 13 haplotype II homozygotes who were N/M, and 13 haplotype II/II heterozygotes who were M/M. Data are shown in TABLE 1. The percentage of $^{A}\gamma^{T}$ in haplotype II/II is about 20% less ($p < 0.001$ by the t test) than the percentage of $\%^{A}\gamma^{I}$ in haplotype I/I. The same conclusion is reached comparing $^{A}\gamma^{T}$ to $^{A}\gamma^{I}$ I/II in heterozygotes ($p < 0.01$).

Our data show that, *in vivo*, the $^{A}\gamma^{T}$-globin gene is expressed at a level which is about 20% less than that of the $^{A}\gamma^{I}$ gene. This is consistent with our hypothesis that the 4-bp deletion at −225 to −222 of the $^{A}\gamma^{T}$ gene decreased gene expression. Data of McDonagh *et al.*[7] and Wall *et al.*[8] show that one or more proteins bind in this region of the γ-globin promoter. One speculates that the 4-bp deletion alters binding of these proteins, which leads to decreased gene expression.

TABLE 1. Expression of $^{A}\gamma^{I}$, $^{A}\gamma^{T}$ and $^{G}\gamma$ in Fetal Hemoglobin of β^{0}-Thalassemia Homozygotes with Haplotypes I/I, I/II, or II/II

	% ± SD			Ratio ± SD		
Haplotype	$^{G}\gamma$	$^{A}\gamma^{I}$	$^{A}\gamma^{T}$	$^{A}\gamma^{I}/^{G}\gamma$	$^{A}\gamma^{T}/^{G}\gamma$	$^{A}\gamma^{T}/^{A}\gamma^{I}$
I/I	50.5 ± 5.9	49.5 ± 5.9	0	1.00 ± 0.24	0	
I/II	55.6 ± 4.0	25.0 ± 4.1	19.4 ± 2.4	0.45 ± 0.11	0.35 ± 0.05	0.79
II/II	61.1 ± 3.2	0	38.9 ± 3.2	0	0.64 ± 0.09	

Is it possible that this reduction in fetal hemoglobin expression exacerbates the clinical course for haplotype II compared to haplotype I homozygotes (both of which have predominantly the β39 nonsense mutation)? Our data show 100/150 chromosomes were haplotype II (67%) and 45/150 were haplotype I (30%). If haplotype II is more severe than haplotype I, one would expect a higher percentage of haplotype II in newborn β-thalassemia homozygotes than in our data from older patients. Data from Masala (unpublished observations) on such newborns showed 12 $^{A}\gamma^{T}/^{A}\gamma^{T}$, 7 $^{A}\gamma^{T}/^{A}\gamma^{I}$, and 2 $^{A}\gamma^{I}/^{A}\gamma^{I}$. While the newborn data are limited, they do not suggest that there is currently significantly more death among haplotype II/II compared to I/I β-thalassemia homozygotes.

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Deletion of a Repeated AG Dinucleotide at the Exon 1–Intron 1 Junction Causes α-Gene Dysfunction without an mRNA Splicing Defect

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In α-thalassemia, the deficiency in α-globin synthesis is most frequently due to deletion of one or more of the normal complement of four α-globin genes. Hemoglobin H disease usually results from the absence of three α genes (genotype $--/-\alpha$). This disorder has a low incidence in black subjects, due to a rarity of the $--/$ haplotype. We have previously reported that the deletion of one of two tandemly repeated AG dinucleotides at the 3′ end of exon 1 near the junction with intron 1 is responsible for a dysfunctional α-globin gene in a black family with α-thalassemia and Hb H disease.[1] The loss of the two nucleotides results in a reading frameshift with the generation of a downstream stop codon in exon 2 at codon 55. We have now found the same genetic lesion in a second, unrelated black family, in which five members have Hb H disease.[2] Because of uncertainty of the effect of nonsense codons on the expression of mRNA, the mutant gene was studied in COS-7 cells, using plasmid-based vectors.[3] A 1.5-kb *Pst* I fragment containing the abnormal gene (α^T) with 5′ and 3′ sequences was ligated to plasmid pLTN1hα after removal of the normal α gene. The α^T and normal α plasmids (together with plasmid pLTN3Bhβ as an internal control) were transfected into COS-7 cells. When production of the normal α and the α^T mRNA was compared to that of the β mRNA, the α/β and α^T/β ratios were 1.6 and 1.8, respectively. The 5′ end of the α^T mRNA was analyzed by primer extension analysis; the extension products mapped to the correct initiation site. The effect of the dinucleotide deletion on the removal of the first intron was studied by S_1 nuclease protection analyses. Correct removal of the intron at both the 5′ donor and 3′ acceptor sites was noted. The 3′ end of the α^T mRNA from transfected COS-7 cells also was analysed by S_1 digestion; the results indicated correct processing.

Our studies showed that the α-thalassemic globin gene is stably expressed at a level comparable to that of a normal α-globin gene when introduced into COS cells, using a plasmid vector. The resultant α-thalassemia mRNA also appeared to have correct 5′ and 3′ ends; and removal of intron 1 proceeded normally at the 5′ and 3′ splice sites, despite the alteration in the consensus sequence near the 5′ donor site. Because the AG dinucleotide is tandemly repeated at the 3′ end of exon 1 of the α-globin gene in the sequence GAGAGgt, the presently described deletion does not destroy the sequence immediately 5 to the invariant gt, which is common to all known introns. This seems a likely explanation for the lack of interference with splicing noted in the present study. This work also suggests that the decrease in the

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steady-state levels of globin mRNA reported with some nonsense mutations[4] may result from effects occurring subsequent to nuclear processing.

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Molecular Characterization of β-Thalassemia Mutations in Chinese[a]

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β-Thalassemia is a relatively common genetic disease in China, with an average incidence of 0.66%.[1] Data from a large survey, involving one million people, 28 provinces and 35 races, showed that the distribution of β-thalassemia is heterogeneous in different parts of China.[1] The Chinese are the most populous ethnic group in the world, with a population of over one billion and with 56 races. Therefore, further characterization of β-thalassemia mutations and demonstration of the distribution of the various β-thalassemia mutants among different regions of China is of much significance for an understanding of the etiology of this disorder in China and for planning a management program. Here we report a study on the molecular characterization of β-thalassemia mutations in various parts of China, using the method of micro-DNA sampling with polymerase chain reaction (PCR) amplification directly from dried blood specimens.

A total of 187 mutant alleles originating from east China (Shanghai, Zhejiang, Jiangsu, Anhui, Jiangxi), southwest China (Sichuan), south China (Guangdong, Guangxi) and other regions (Hubei, Hunan) were analyzed. Tested blood samples were collected on Xinhua #3 filter papers, and the dried blood samples were mailed to the authors' laboratory. They were then subjected to a direct PCR procedure, without prior DNA extraction, according to the method we described previously,[2] using two pairs of oligomer primers which amplify 601 and 422 bp of β-globin DNA sequences, respectively. The amplified DNA was dot-blot hybridized with ten types of Chinese β-thalassemia allele-specific oligonucleotide (ASO) probes.[3]

The distribution of various types of β-thalassemia alleles is shown on the map according to the places from which the tested mutant alleles originated (FIG. 1). The results indicated several points (TABLE 1): (1) Different types of β-thalassemia mutations prevail in different regions. The commonest types in south China (Guangdong, Guangxi) are a frameshift at codons 41/42 and a C→T transition at IVS-2 nucleotide (nt) 654. The most frequent types in southwest China (Sichuan) are a nonsense mutant at codon 17 and the IVS-2 nt 654 mutation; while in east China, the predominant mutant alleles are frameshifts at codons 41/42 and 71/72. (2) As expected, all the mutants from south China are of known types; and the data are very similar to those of other investigators. In contrast, a relatively higher proportion of unknown (i.e., not yet characterized) mutations are found in the southwest and east parts of China.

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TABLE 1. Type and Frequencies of β-Thalassemia Genes in Chinese

Area	nt −29 A→G	nt −28 A→G	Codons 14/15 +G	Codon 17 A→T	IVS-1 nt 1 G→T	IVS-1 nt 5 G→C	Codons 41/42 −4 bp	Codon 43 G→T	Codons 71/72 +A	IVS-2 nt 654 C→T	Unknown	Total
Guangdong												
n	0	3	0	3	0	1	16	1	2	8	0	34
(%)	(0)	(8.8)	(0)	(8.8)	(0)	(2.9)	(47.1)	(2.9)	(5.9)	(23.6)	(0)	(100)
Guangxi												
n	0	3	0	4	1	0	11	0	2	6	1	28
(%)	(0)	(10.7)	(0)	(14.3)	(3.6)	(0)	(39.3)	(0)	(7.1)	(21.4)	(3.6)	(100)
Sichuan												
n	0	1	0	8	0	0	4	0	1	5	5	24
(%)	(0)	(4.2)	(0)	(33.3)	(0)	(0)	(16.7)	(0)	(4.2)	(20.8)	(20.8)	(100)
East China												
n	0	3	0	4	0	6	22	0	16	8	6	65
(%)	(0)	(4.6)	(0)	(6.2)	(0)	(9.2)	(33.8)	(0)	(24.6)	(12.3)	(9.3)	(100)
Other												
n	0	1	1	4	0	2	9	0	3	7	9	36
(%)	(0)	(2.8)	(2.8)	(11.1)	(0)	(5.6)	(25.0)	(0)	(8.3)	(19.4)	(25.0)	(100)
Total												
n	0	11	1	23	1	9	62	1	24	34	21	187
(%)	(0)	(5.9)	(0.5)	(12.3)	(0.5)	(4.8)	(33.2)	(0.5)	(12.8)	(18.2)	(11.3)	(100)

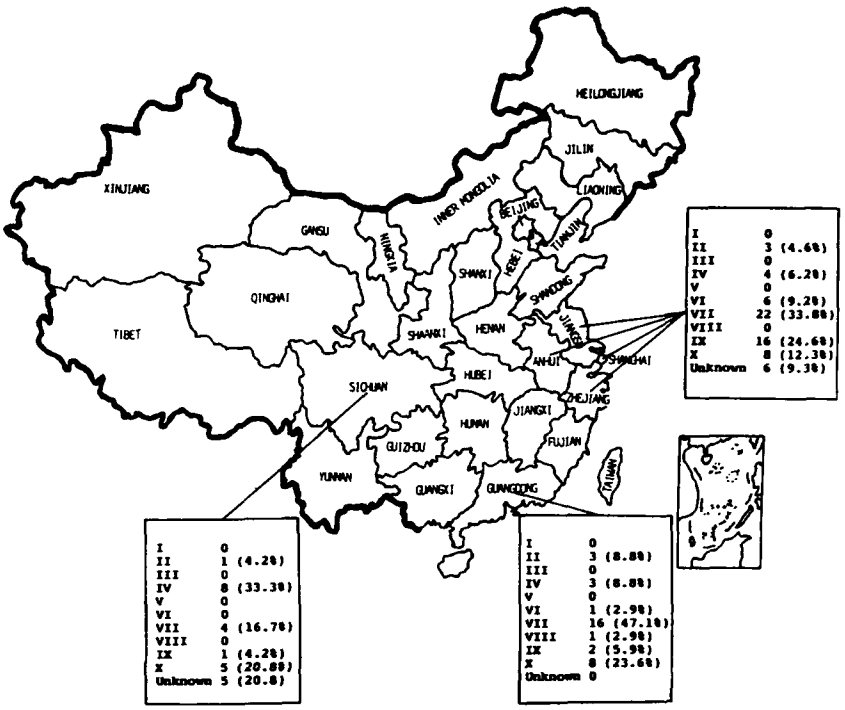

FIGURE 1. Distribution of various types of β-thalassemia mutations in China. *Roman numerals* represent the different mutant types, listed in their order, 5′ to 3′, in the β-globin gene: **(I)** −29 A→G, **(II)** −28 A→G, **(III)** codons 14/15 +G, **(IV)** codon 17 A→T, **(V)** IVS-1 nt 1 G→T, **(VI)** IVS-1 nt 5 G→C, **(VII)** codons 41/42 −4 bp, **(VIII)** codon 43 G→T, **(IX)** codons 71/72 +A, **(X)** IVS-2 nt 654 C→T.

The method of micro-DNA sampling from dried blood, followed by direct PCR amplification, greatly eases sample transport and simplifies the procedure of DNA analysis. Its application provides a new resource for patient diagnosis and molecular screening among the general population.

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Screening for β-Thalassemia in Asian Indians by the Amplification-Refractory Mutation System

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The amplification-refractory mutation system (ARMS) has been applied to detect nine mutations believed to cause β-thalassemia in an Asian Indian population. The basis of this system is that oligonucleotides with a mismatched 3′ end will not function as amplimers in the polymerase chain reaction (PCR) under appropriate conditions. Genotyping is possible by visualization of the PCR product by agarose gel electrophoresis and ethidium bromide staining, making this a simple, reliable, non-isotopic method for carrier detection and prenatal diagnosis.

Oligonucleotides were designed corresponding to both the wild-type and mutant alleles for each of the nine mutations, with the mismatch at the 3′ end. To improve the specificity, another mismatch was introduced at a position four bases away from the 3′ end. Initially, each of these allele-specific amplimers was designed to be the downstream primer to be used with a common upstream primer (primer A). However, for seven of the oligonucleotides it was necessary to reverse this order and couple them with a common downstream primer (primer B). Primers C and D, which amplify an 861-bp fragment from further downstream along the β-globin gene, were used as the internal control for the PCR. An additional advantage of this pair of internal-control amplimers is that they directly detect the presence of the 619-bp deletion found in this population. The primer sequences are shown in TABLE 1.

The PCR was performed in a 25-μl reaction volume, with 100 ng of genomic DNA, 5 pmol of each primer, 0.5 units of *Taq* polymerase, 30 μ*M* of each dNTP in 10 m*M* Tris-HCl, pH 8.4, 50 m*M* KCl, 1.5 m*M* $MgCl_2$, and .01% gelatin. The amplification regimen consisted of 25 cycles of denaturation at 93 °C for 1 min followed by annealing and extension at 66 °C for 2 min, with a final extension step at 66 °C for 3 min in the last cycle. 20 μl of the PCR product was electrophoresed on an ethidium bromide–stained 3% agarose gel.

TABLE 1. Primers Used for Detection of Asian Indian β-Thalassemia Mutations by ARMS

Mutation	Type[a]	Primer Sequence
Allele-specific[b]		
IVS-1 nt 5 (G→C)	N	5′-CTCCTTAAACCTGTCTTGTAACCTTGTTAC
	M	5′-CTCCTTAAACCTGTCTTGTAACCTTGTTAG
IVS-1 nt 1 (G→T)	N[c]	5′-GATGAAGTTGGTGGTGAGGCCCTGGGTAGG
	M	5′-TTAAACCTGTCTTGTAACCTTGATACGAAA
FS codons 8/9 (+G)	N	5′-CCTTGCCCCACAGGGCAGTAACGGCACACT
	M	5′-CCTTGCCCCACAGGGCAGTAACGGCACACC
FS codons 41/42 (−CTTT)	N	5′-GAGTGGACAGATCCCCAAAGGACTCAAAGA
	M	5′-GAGTGGACAGATCCCCAAAGGACTCAACCT
Nons codon 15 (C→A)	N[c]	5′-TGAGGAGAAGTCTGCCGTTACTGCCCAGTG
	M[c]	5′-TGAGGAGAAGTCTGCCGTTACTGCCCAGTA
FS codon 16 (−C)	N[d]	5′-TCACCACCAACTTCATCCACGTTCACGTTG
	M	5′-TCACCACCAACTTCATCCACGTTCACGTTC
nt −88 (C→T)	N[c,d]	5′-TCACTTAGACCTCACCCTGTGGAGCCTCAC
	M[c]	5′-TCACTTAGACCTCACCCTGTGGAGCCTCAT
CAP 1 (A→C)	N[c,d]	5′-ATAAGTCAGGGCAGAGCCATCTATTGGTTA
	M[c]	5′-ATAAGTCAGGGAAGAGCCATCTATTGGTTC
IVS-1, −25 (−25 bp)	N[d]	5′-CCAGCAGCCTAAGGGTGGGAAAATAGACCA
	M	5′-CCAGCATAGGCAGAGAGAGTCAGTGCCTAT
Common		
Upstream	A	5′-ACCTCACCCTGTGGAGCCAC
Downstream	B	5′-CCCCTTCCTATGACATGAACTTAA
Internal control	C	5′-CAATGTATCATGCCTCTTTGCACC
	D	5′-GAGTCAAGGCTGAGAGATGCAGGA

[a]N, wild-type primer; M, mutant primer.
[b]nt, nucleotide; FS, frameshift; Nons, nonsense.
[c]Coupled with common downstream primer B; all other allele-specific primers were coupled with common upstream primer A.
[d]Normal primers (N) for these mutations have not yet been tested, due to the lack of a homozygote.

TABLE 2. Frequency of Mutations amongst Asian Indians

Mutation[a]	*n*	%
IVS-1 nt 5	115	33.5
FS codons 8/9	75	21.9
619-bp deletion	59	17.2
FS codons 41/42	37	10.8
IVS-1 nt 1	36	10.5
Nonsense codon 15	8	2.3
FS codon 16	1	0.3
CAP 1	1	0.3
Uncharacterized	11	3.2
Total	343	100

[a]nt, nucleotide, FS, frameshift.

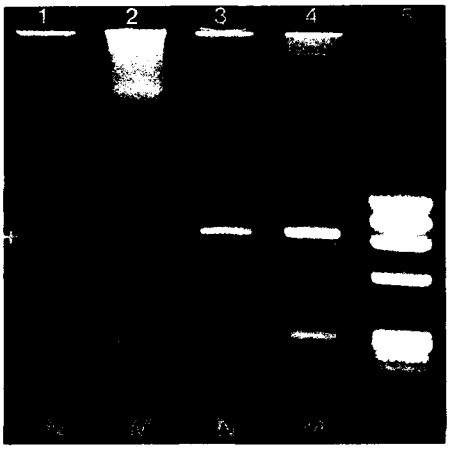

FIGURE 1. Ethidium bromide-stained agarose gel electrophoresis of PCR products showing the detection of the IVS-1 nt 5 mutation. **(Lanes 1 and 2)** Normal sample amplified by normal (N) and by mutant (M) allele-specific primers, respectively; **(lanes 3 and 4)** same analysis for a sample homozygous for the IVS-1 nt 5 mutation. **(Lane 5)** Pattern for *Hae* III-digested ϕX174 standard fragments. Position of band amplified by primers C and D, as a positive internal control, is indicated (+).

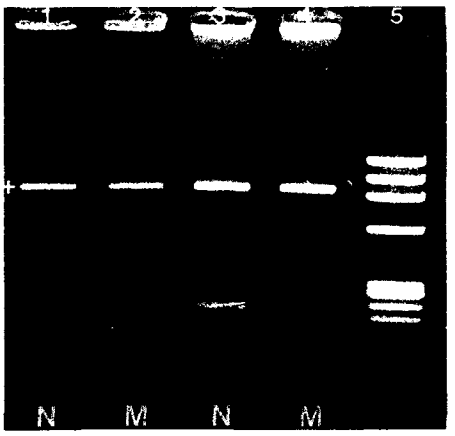

FIGURE 2. Ethidium bromide-stained agarose gel electrophoresis of PCR products showing the detection of the frameshift codons 8/9 mutation. **(Lanes 1 and 2)** Sample homozygous for the frameshift mutation amplified with normal (N) and mutant (M) allele-specific primers, respectively; **(lanes 3 and 4)** same analysis for a normal sample. **(Lane 5)** Pattern for *Hae* III-digested ϕX174 standard fragments. Position of band amplified by primers C and D, as a positive internal control, is indicated (+).

This system has been used to study 343 Asian Indian β-thalassemia carriers from various regional and ethnic groups. TABLE 2 depicts the frequency of different mutations, and FIGURES 1 and 2 show representative gel patterns. We hope to use this method to characterize the mutations of β-thalassemia carriers from the different Indian regions, with a view to constructing a "mutation map" of the Indian subcontinent.

α-Globin Variants in Southeast Asians in Hawaii[a]

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Families of Southeast Asian and other ethnicities in Hawaii were screened for the thalassemias; in all, 3,198 Southeast Asians were screened. An ethnic outreach worker helped to recruit Laotians; physicians were invited to refer anemic patients with their relatives. Red cell indices were used to identify microcytosis, fluorometric zinc protoporphyrin to rule out iron deficiency, cellulose acetate or isoelectric focusing electrophoresis to look for hemoglobin (Hb) variants, and Hb A_2 microchromatography to look for β-thalassemia heterozygotes.[1] Provisional diagnoses were based when possible on intrafamily comparisons. All subjects over age 6 had blood taken for α-globin gene DNA analyses by Southern blotting with ζ and α probes, to look for α-gene deletions, and for polymerase chain reaction/slot blot hybridization to detect the Hb Constant Spring mutation.[2] Family DNA polymorphisms helped to confirm diagnoses.

The samples were from 650 Laotians, 548 Chinese, 325 part-Chinese, 158 Filipino-Chinese, 1305 Filipinos, 208 part-Filipinos, and 480 people of other races. Provisional diagnoses are shown in TABLE 1 and FIGURE 1.

TABLE 1. Provisional Thalassemia Diagnoses by Ethnicity

Diagnosis[a]	Chinese	Filipino	Laotian	Others	Total
Iron deficient	11	90	7	16	124
Normal	519	836	208	322	1885
Borderline	48	59	44	22	173
α_2-Thal heterozygote	36	81	63	15	195
α_1-Thal heterozygote	128	449	30	38	645
Hb H disease	7	39	10	5	61
β-Thal heterozygote	36	81	63	15	195
Hb AC heterozygote				2	2
Hb AD heterozygote		2		1	3
Hb AE heterozygote	4	13	249	13	279
Hb EE homozygote			31	1	32
Hb AS heterozygote				5	5
Hb H/Constant Spring			4	1	5
α_1-Thal homozygote	2	3	1		6
β-Thal homozygote		1		2	3
Hb E/β-thal		1	2		3
Total	791	1655	712	458	3616

[a]Thal, thalassemia.

[a]This work was supported by U.S. Public Health Service MCH Grant MCJ-151002.

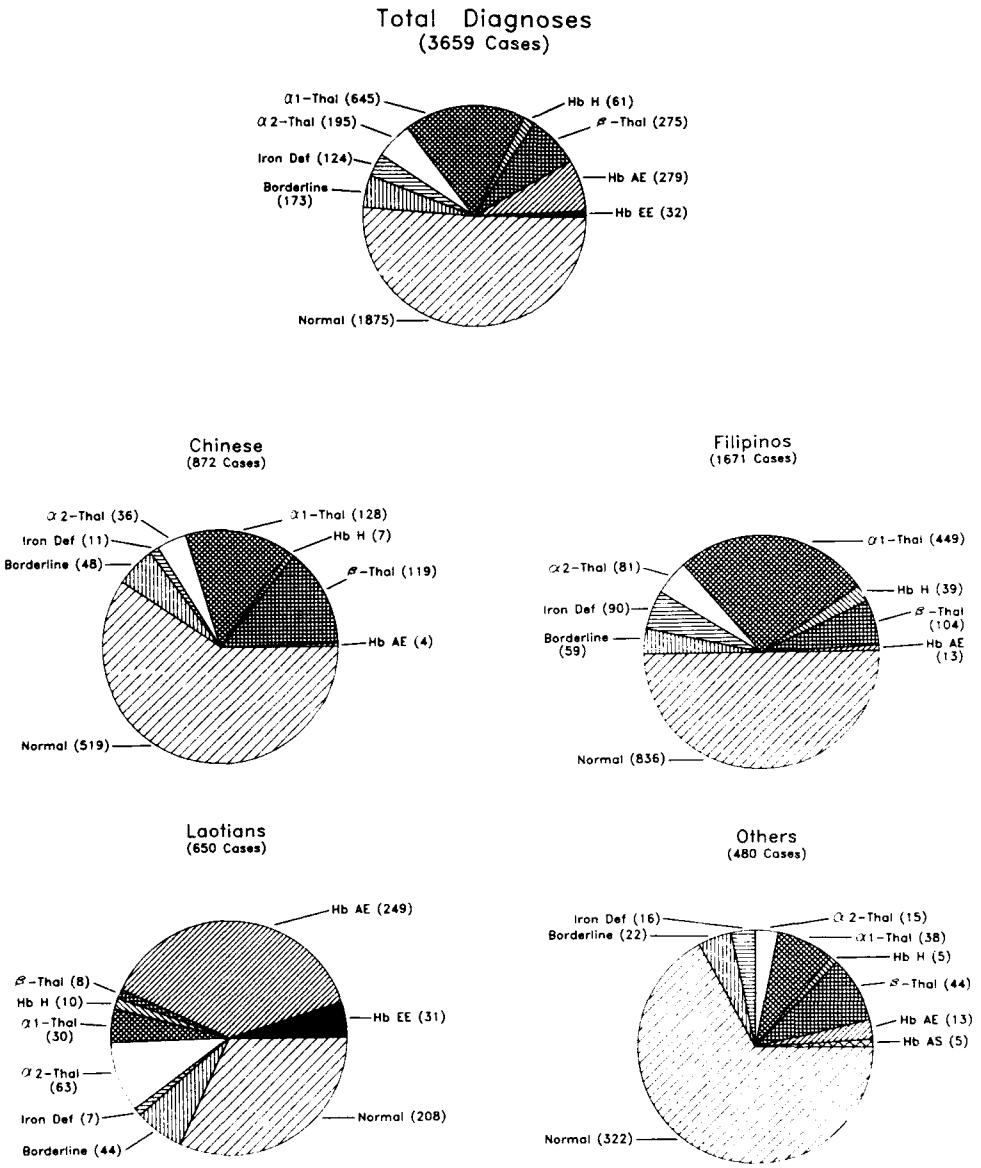

FIGURE 1. Provisional diagnoses for Southeast Asians and people of other ethnicities screened for thalassemias in Hawaii. Thal, thalassemia; Iron Def, iron deficiency.

So far, 796 diagnoses have been confirmed or revised by DNA analyses. The distributions of abnormal genotypes among all people tested except for the Laotians are increased by ascertainment bias, except that many "silent" single-deletion α_2-thalassemia heterozygotes must remain among those provisionally diagnosed as

normal. The numbers of subjects with the more severe α and β variants are unlikely to be biased relative to one another, even if the absolute frequencies of these variants may be biased.

In Hawaii, heterozygotes for the α_1-thalassemia double deletions and compound heterozygotes with Hb H disease have high prevalence, especially among the Filipinos, who have more of the $(----^{Fil})$ total deletions than of the Southeast Asian $(--^{SEA})$ double deletions that predominate in the other ethnic groups. The β-thalassemia variants are more prevalent among the Chinese than among the Filipinos, and less common in Laotians. Many Southeast Asian families have mixed heterozygotes with both α and β variants.

These data confirm that Southeast Asian populations have high frequencies of both α-globin and β-globin gene variants,[3] and many couples will be at risk for having severely affected children.[4] Population heterozygote screening can greatly reduce the number of births of severely affected infants by genetic counseling and the offer of fetal testing. Effective screening and accurate diagnoses require techniques that can identify specific α-globin gene variants, even in the presence of β-globin variants.

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Thalassemia in the Southeastern Part of Sicily[a]

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Over the centuries, Sicily has been at the crossroad of several ethnic invasions and has thus become the melting pot of various thalassemic syndromes, further expanded by the past history of malarial endemicity. Given this historical background and the heterogeneity of DNA haplotypes so far observed,[1] it is expected that the spectrum of β-thalassemia mutations in Sicily must be large and that the heterozygosity for mutations should be very high. With the advent of the technic of *in vitro* DNA amplification by the polymerase chain reaction (PCR), direct examination of the mutations now becomes simple, rapid and, hence, applicable to large series of samples. We report here our studies on the molecular characterization of β-thalassemia mutations in the southeastern part of Sicily.

A total of eighty-nine adult patients from the southeastern part of Sicily, routinely followed at the Center for Thalassemia in Catania, and presenting a clinical continuum from a severe transfusion-dependent Cooley's anemia to an extremely mild form (the latter being discovered by chance), were examined for the β-globin gene defect.

Five different mutations (β^+ IVS-1 nt 6, β^0 nonsense codon 39, β^+ IVS-1 nt 110, β^+ IVS-2 nt 745, and β^0 IVS-1 nt 1) account for more than ninety percent of the thalassemic chromosomes in this part of Sicily, as shown in TABLE 1. Among them, the most prevalent, the IVS-1 nt 6 mutation, is found on four different haplotypes all belonging to the same 3′ subset of polymorphisms (framework 3). Hence it is explainable by a recombination event in the hot spot region 5′ of the β gene. A similar explanation is valid for the β^0 nonsense codon 39 mutation found on two different haplotypes. The prevalence of these mutations alone does not account for their dispersion on different haplotypes, because the IVS-1 nt 110 mutation, which is more prevalent than the β^0 nonsense codon 39 mutation, is restricted to a single haplotype. Hence, as suggested already by Di Marzo *et al.*,[2] the IVS-1 nt 110 mutation could have been introduced into Sicily, probably by Greek-Cypriot migration, more recently than were the other two mutations presumptively of Italian origin.

[a]This work was done in an INSERM–CNR joint research program.
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TABLE 1. The Spectrum of β-Thalassemia Mutations in the Southeastern Part of Sicily

Haplotypes	*n*	Mutation[a] IVS-2 nt 1	IVS-1 nt 110	NS β^0 39	IVS-1 nt 1	IVS-1 nt 6	IVS-2 nt 745	N.I.
I	59	—	46	9	—	—	—	4
II	32	—	—	32	—	—	—	—
III[b]	3	3	—	—	—	—	—	—
V	8	—	—	—	5	—	—	3
VI	52	—	—	—	—	52	—	—
VI[s]	3	—	—	—	—	3	—	—
VII	13	—	—	—	—	1	11	1
x	7	—	—	7	—	—	—	—
y	1	—	—	—	—	1	—	—
Total	178	3	46	48	5	57	11	8
(%)	(100)	(1.5)	(26)	(27)	(3)	(32)	(6)	(4.5)

[a]NS β^0 39, β^0 nonsense codon 39; N.I., not identified.
[b]Non-deletion HPFH individuals: β-thalassemia without anemia.

TABLE 2. 5′ Subset Restriction Site Polymorphisms of Haplotype III–associated Framework 2

Framework 2	Restriction Fragment *Hinf* I 5′ β	*Rsa* I 5′ β	*Ava* II β	*Hinf* I 3′ β	*Bam*H I 3′ β
HPFH-associated Sicilian	−	+	+	−	−
Other	+	−	+	−	−

None of these mutations are exclusive to a mild or a severe clinical phenotype, although two-thirds of the thalassemia intermedia individuals carry at least one β gene with the IVS-1 nt 6 mutation.

The most interesting aspect of this study is the observation of two cases (in patients 70 and 40 years old, respectively) of homozygous β-thalassemia without anemia. Both of them carry the IVS-2 nt 1 mutation either in homozygous or in compound heterozygous state with a Sicilian δβ-thalassemia. Although these forms are known to cause transfusion-requiring anemia in other population groups, the high–Hb F phenotype associated with the haplotype III chromosome (TABLE 1) rendered the clinical course extremely mild. These framework 2 chromosomes differ from other known framework 2 chromosomes by their polymorphism of the 5′ β-globin gene region (TABLE 2). The significance of this finding with respect to the hereditary persistence of fetal hemoglobin (HPFH) phenotype is not yet clear. It is of interest to note the existence of a similar clinical phenotype reported in a Guyanese individual.[3]

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Intriguing Prevalence of Benin-Type Sickle Cell Gene on Atypical Haplotypes in Sickle Cell/β-Thalassemia But Not in Sickle Cell Anemia in Sicily[a]

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The sickle cell mutation in Sicily is found in linkage disequilibrium with the Benin haplotype.[1,2] Since this haplotype is not found on β^A or β^{Thal} chromosomes in this population, it is believed that the β^S gene was introduced into Sicily from central West Africa and has been maintained by malarial selective pressure. No recombination of the Benin haplotype was observed in more than 50 unrelated Sicilian sickle cell patients or among β^S chromosomes of 20 unrelated sickle cell trait individuals. Here, we report an intriguing feature of the β^S chromosomes in Sicilian sickle cell/β-thalassemia patients, where they appear to have recombined more frequently than in other contexts.

By family studies, we have determined the DNA restriction haplotypes of β^S and β^{thal} chromosomes in 20 sickle cell/β-thalassemic patients from the Thalassemia Center in Catania. As expected, all the β^S genes were found to be associated with a 13-kb *Hpa* I polymorphic fragment. However, complete haplotype analysis and segregation pattern studies in families revealed that four out of these 20 β^S chromosomes bore a haplotype different from that of the Benin type (TABLE 1). Since the observed differences affect the 5′ subhaplotype only, association of these four β^S genes with atypical haplotypes should be the result of meiotic recombinations upstream from the β-globin gene. Combining our data with the nine cases reported by Sammarco *et al.*[2] reveals that such recombinations are amazingly frequent only when the β^S gene is associated with β-thalassemia. We have explored the region of a potential recombination hot spot by studying the polymorphic *Hinf* I site 5′ to the β^S gene[3] and two other polymorphic sites (*Rsa* I and *Hinf* I 3′ β) downstream from this region.[4] Our results indicate that the cross-over has taken place 5′ to the *Rsa* I site (FIG. 1). We do not have a clear explanation for this high rate of recombination of β^S chromosomes in sickle cell/β-thalassemia patients. It is possible that the flow of sickle cell genes in Sicily has occurred in several waves and that β^S chromosomes of

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TABLE 1. β-Globin Gene Restriction Fragment Length Polymorphism (RFLP) Haplotypes in Sicilian Sickle Cell β-Thalassemia

Observed Haplotype Combinations[a]	*n*	Thalassemic Mutation[b]
β^{thal} −−−−++/−−−−++ β^{S*}	1	IVS-1 nt 110
β^{thal} −−−−++/+++−+++ β^{S*}	1	ND
β^{thal} ++−+++/−−−−++ β^{S*}	1	NS β^0 39
β^{thal} ++−+++/++−−++ β^{S*}	1	NS β^0 39
β^{thal} +−+++−/−−−+++ β^S	1	IVS-2 nt 1
β^{thal} −−−−++/−−−+++ β^S	3	IVS-1 nt 110
β^{thal} ++−−−+/−−−+++ β^S	3	IVS-1 nt 6
β^{thal} ++−+++/−−−+++ β^S	9	NS β^0 39

[a]The RFLP sites are as described by Orkin *et al.*[5] Their distribution on the β^{thal} and β^S chromosomes was determined by family study. β^{S*} indicates that the β^S gene is on atypical haplotypes.

[b]ND, not determined; NS β^0 39, β^0 nonsense codon 39.

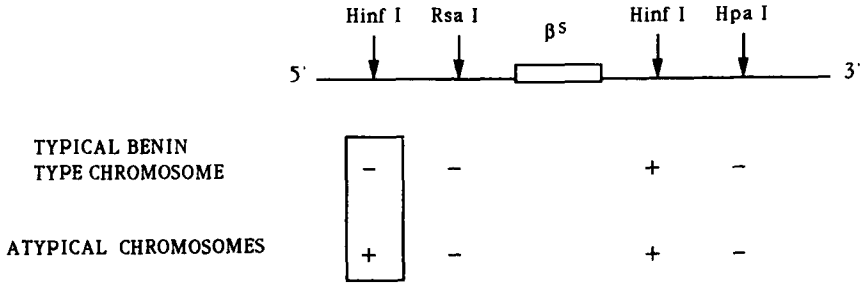

FIGURE 1. Mapping of the probable cross-over region in the β^S chromosome.

the most ancestral flow have had time not only to associate with the locally prevalent β-thalassemic chromosomes but also to recombine with them. Precise investigation of population history might help to clarify this intriguing aspect of the β^S gene in sickle cell/β-thalassemia in Sicily.

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Increase with Age in the Prevalence of β-Thalassemia Trait among Sicilians[a]

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The clinical implications of heterozygosity for β-thalassemia are largely unknown. On the one hand, the high prevalence of β-thalassemia carriers in areas with the highest incidence of malaria in the past has given rise to the hypothesis that β-thalassemia heterozygotes are more resistant to malaria than are normal homozygotes.[1,2] It was supposed that thalassemic erythrocytes were less suitable as hosts for the development of the plasmodium because of their increased susceptibility to peroxide damage, their lesser content of hemoglobin or their elevated surface: volume ratio, but up to now none of these hypotheses has been confirmed; and the evidence for a decreased mortality from malaria of the β-thalassemic heterozygotes still remains indirect.[1,2] On the other hand, it has been suggested that β-thalassemia carriers are more prone than normal homozygotes to other diseases, such as arthropathy,[3-6] aseptic necrosis[7] and complications of pregnancy such as severe anemia,[8-10] but in these reports also the evidence is weak and anecdotal. Recently, Canella *et al.*[11] studied the "biological performance" of β-thalassemia carriers in an area from which malaria had been eradicated. In a population from a small village of Northern Italy with a high prevalence of thalassemia, which was screened in 1956 by Silvestroni and Bianco and assessed again in 1981–1985, i.e., 35 years after malaria eradication, they did not find a significant difference in the mortality rate between microcythemics and normocythemics. Therefore they concluded that there were no differences between the biological performance of thalassemics and of normals in their series.

We have explored a different approach to the problem. Since a detrimental effect on the biological performance of β-thalassemia heterozygotes should produce a lower prevalence of the mutant gene in the elderly, and, on the contrary, a favorable selection should increase the β-thalassemia gene frequency with age, we studied at the same time the prevalence of β-thalassemia carriers among children (unexposed to malaria) and among elderly people (exposed to malaria in their youth) in four villages of our region from which malaria had been eradicated in the late 1940s. We found that the prevalence of thalassemia was significantly and consistently higher in the elderly group than in the children. These results suggest that in the past malaria played an important role in determining the high prevalence of β-thalassemia in this area, and that, as suggested by Canella *et al.*,[11] after malaria eradication, the survival

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of β-thalassemia heterozygotes has remained comparable to that of normal homozygotes.

Four villages of the southeastern area of Sicily, where malaria was endemic until the late 1940s, and for which there is substantial archeological and historical evidence demonstrating the presence of malaria from about 600 B.C.,[12] were selected for the study: Bronte (resident population 21,218), Grammichele (residents, 13,965), Niscemi (residents, 26,228), and Regalbuto (residents, 9,677). These villages all have the same socioeconomic structure, mainly based on agriculture, and are located in the hinterland, 40 to 60 kilometers from the sea. Since the four villages have modest socioeconomic conditions, immigration has never occurred.[12,13]

From 1984 to 1987 we studied a total of 4032 subjects: 2527 children (1107 boys and 1420 girls), aged 6 to 16 years, randomly selected in the schools, and 1505 elderly people (673 males and 832 females), aged 60 to 90 years, screened in homes for the old, hospices, and clubs. All the subjects studied were born in one of the above-mentioned villages.

TABLE 1. Prevalence of β-Thalassemic Heterozygotes According to Age in Four Sicilian Villages

			Distribution by Age				
	All		< 16 Years		> 60 Years		
Village	Rate	%	Rate	%	Rate	%	χ^2
Bronte	58/880	6.59	28/480	5.83	30/400	7.50	0.98
Grammichele	99/1556	6.36	56/969	5.78	43/587	7.33	1.47
Niscemi	34/518	6.56	17/366	4.64	17/152	11.18	7.49[a]
Regalbuto	53/1078	4.92	31/712	4.35	22/366	6.01	1.42
All villages	244/4032	6.05	132/2527	5.22	112/1505	7.44	8.16[a]
All except Niscemi	210/3514	5.98	115/2161	5.32	95/1353[b]	7.02[b]	4.20[a]

[a] $p < 0.01$.
[b] In subjects > 75 years old: 25/206 (12.14%).

Cell counts were performed on heparinized blood using a Coulter ZB1 cell counter, and hemoglobin was determined in a Coulter hemoglobinometer. Hemoglobin electrophoresis was performed on Titan III cellulose-acetate plates at pH 8.4 in Tris-EDTA-borate buffer, and fetal hemoglobin was measured as described by Betke *et al.*[14] Hemoglobin A_2 was measured by the microchromatographic technique of Huisman *et al.*[15] Serum ferritin and protoporphyrin were also measured to rule out iron deficiency. A diagnosis of β-thalassemia trait was made in the presence of microcytosis, increased levels of Hb A_2, and normal levels of ferritin and protoporphyrin.

The results are given in TABLE 1. The overall prevalence of β-thalassemia carriers in the population under study was 6.05% (244/4032). The prevalence ranged from 4.92 in Regalbuto to 6.59 in Bronte.

In all four villages the prevalence of β-thalassemia carriers was lower in the subjects 6–16 years old (5.22%, range 4.35% to 5.83%) and higher in the 60–90-year-old group (7.44%, range 6.01% to 11.18%). The difference was statistically significant (χ^2, 8.16; $p < 0.01$). The difference was greatest in Niscemi (4.64% versus 11.18%), but it remained significant at the $p < 0.01$ level even when only the other three villages were considered as a whole (5.32% versus 7.02%). By further subdivid-

ing the subjects over 60 years into two groups according to age (i.e., 60–75 and 76–90 years), the carriers were 87/1299 (6.7%) and 25/206 (12.14%), respectively (χ^2, 7.635; $p < 0.01$).

Our study revealed a clear-cut age effect on the prevalence of β-thalassemia trait in our population. Among the subjects over 60 years old, the prevalence of the trait was significantly higher than in subjects 6–16 years old. Among the elderly subjects, the prevalence was lower in the 60–75-year-old group and higher in the 76–90-year-old group.

We believe that the most important, if not the only, cause of this difference is the selective action of malaria. In fact, since in our area endemic malaria was eradicated about 40 years ago, the subjects over 60 years of age were exposed to malaria for at least 20 years, and those over 70 years of age for at least 35 years; it is therefore likely that during those years more normal homozygotes than β-thalassemia heterozygotes succumbed to malaria. The epidemiology of malaria in Sicily was characterized by the concomitant presence of *Plasmodium falciparum* (about 20%) and *P. vivax* (about 80%).[16] Our observation thus suggests that even *P. vivax* may play a significant role as a selective factor.

The high prevalence of β-thalassemia carriers in the older population shows that, as suggested by Canella *et al.*,[11] the life expectancy of β-thalassemia heterozygotes is equal, if not better, than that of normal homozygotes. However—since malaria has been eradicated from our area—if this difference in the prevalence of β-thalassemia trait between young and old people is entirely due to malaria, from now on the prevalence of β-thalassemia carriers in our population should not increase with age and should remain around 5.22% (as in the 6–16-year-old group) in the entire population. However, it is possible that in addition to malaria, possession of the β-thalassemia trait resulted in protection against other, up to now unrecognized, disease states over the centuries. There are a few suggestions in the literature that β-thalassemia trait may be a protective factor against cardiovascular disease, a most frequent cause of death in humans. For example, recently Crowley *et al.*[17] demonstrated that the prevalence of myocardial infarction, which is a frequent cause of death, in subjects with β-thalassemia trait was statistically lower than that in controls. Our previous study on 107 β-thalassemia trait adults (20–50 years of age) revealed no alterations in blood pressure, in heart functioning and in plasma lipids (cholesterol and triglycerides), while the packed cell volume (PCV) and blood viscosity were significantly lower than they were in normal controls.[18] Similar findings in cholesterol levels were reported by Maioli *et al.*[19] There are findings which show that a high hemoglobin value is a "risk factor" in myocardial infarction.[20,21]

The intervention of recently introduced additional factors may increase the frequency of the β-thalassemia gene in the long run. These factors are the increasing availability of antenatal diagnosis (which encourages procreation by the couples at risk), the improvement of conventional therapy, and the introduction of bone marrow transplantation (both of which will enable thalassemic homozygotes to procreate). In the future, somatic gene therapy might also contribute to this increase.

In summary, then, we have studied the prevalence of β-thalassemia trait in two age groups (2,527 subjects less than 16 years old and 1,505 subjects aged more than 60 years) in four Sicilian villages from which malaria had been eradicated in the 1940s. Significantly more carriers were found in the elderly group (7.44% versus 5.22%, $p < 0.01$). This result supports the notion that in the past malaria played a very important role in establishing the high prevalence of β-thalassemia in this area.

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Sickle Cell Thalassemia in Greece

Identification and Contribution of the Interacting β-Thalassemia Gene

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The levels of Hb A in compound heterozygotes of Hb S and β-thalassemia reflect the activity of the interacting β-thalassemia gene. In fact, it is on this basis that the denominations β^0 (no Hb A), β^+ (low Hb A) and β^{++} (relatively high Hb A) were coined. The present communication aims to correlate the phenotypes of 40 Greek patients who have Hb S/β-thalassemia with the underlying molecular thalassemic defects in comparison to 30 patients who have homozygous Hb S disease.

Hematological and biochemical values were determined by standard methods. Molecular analysis was carried out by hybridization with a battery of oligonucleotides identifying the thalassemic mutations prevailing in Greece, after selective β gene amplification by the polymerase chain reaction (PCR). Search for the −158 polymorphism was also done by PCR, using suitable oligomers and the *Xmn* I enzyme. The number of α genes was determined by standard *Bgl* II and *Eco*R I gene mapping.

Red blood cell (RBC) heterogeneity was studied either by the RDW value[b] (Coulter counter) or by centrifugation of RBC suspensions in a series of microhematocrit tubes, each containing a layer of phthalate esters of increasing specific density which equilibrated at various heights within the suspension and separated the red cell column into two parts, one lighter and another more dense than the ester in each tube. Results were plotted on paper and, from the graph, the arbitrary R60 × 10^{-4} value was determined.

The results of these studies are summarized in TABLE 1. The values for the parameters measured, despite their large variance, confirm the expected intergroup increments of Hb A; but they also show a parallel, statistically significant increase of the total hemoglobin level, along with a reciprocal decrease of the mean corpuscular hemoglobin (MCH) and, interestingly, of Hb F. Clinical severity was not different among the groups, although the condition was extremely mild in some extreme cases with high Hb A or high Hb F.

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[b] RDW: red cell volume distribution width, expressing the degree of anisocytosis.

TABLE 1. Values of Main Hematological and Biochemical Parameters in Compound Heterozygotes with Hb S and β-Thalassemia and in Homozygotes with Hb S

	Value (mean ± SD)			
	Compound Heterozygotes of Hb S[a]			Homozygotes
Parameter	β^0 thal	β^+ thal	β^{++} thal	β^S
n	18	19	3	30
Hemoglobin (g/dl)	8.4 ± 1.25	10.4 ± 1.42	11.1–13.0	9.3 ± 1.10
MCH (pg)	25.0 ± 1.85	23.1 ± 1.68	20.6–23.7	33.5 ± 2.90
Reticulocytes (%)	16.7 ± 7.30	9.1 ± 5.90	10.0–12.0	17.6 ± 7.40
Hb A (%)	0	10.2 ± 3.47	10.0–25.0	0
Hb F (%)	10.2 ± 4.10	5.0 ± 2.20	1.0–4.8	7.4 ± 4.10
RBC heterogeneity ($R60 \times 10^{-4}$)	113 ± 27	81 ± 21	66	112 ± 56
Molecular defects	β^0 −39 IVS-1 nt 1 IVS-2 nt 1	IVS-1 nt 110 IVS-2 nt 745	IVS-1 nt 6	

[a]thal, thalassemia.

RBC were less heterogeneous in the group with high Hb A and markedly heterogeneous in the β^0-thalassemia/β^S group, especially when the latter was associated with high Hb F. The β^S/β^S patients gave a similar pattern. This finding may reflect the genetically determined presence of equal amounts of Hb A in all RBC, in contrast to the epigenetic uneven "addition" of Hb F over the RBC population.

Hematological Parameters for α- and β-Thalassemia Variants[a]

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In a Hawaii thalassemia (Thal) program,[1] 3616 subjects were screened. DNA analyses of the α-gene region[2,3] have been completed in 796; Southern blotting was used for α-gene deletions[4] and polymerase chain reaction/slot blot hybridization for the Hb Constant Spring mutation[5] (TABLE 1).

The red cell indices of the subjects with known α DNA genotypes were reviewed, to assess the validity of using microcytosis to screen for the α^{Thal} and β^{Thal} heterozygotes.[1,4] A total of 486 α-1 and 263 β-thalassemia variants was examined.

The data for mean corpuscular volume (MCV) are presented by diagnosis, both as Gaussian curves and as frequencies in increments of 5 fl (FIG. 1). MCV and mean corpuscular hemoglobin (MCH) were highly informative for distinguishing α^{Thal} and β^{Thal} heterozygotes and Hb E homozygotes from apparent normals.[1,4] Decreases in MCV and MCH correlated well with the number of deleted α genes in the α^{Thal}

TABLE 1. 796 β-Globin and α-Globin Genotypes

	$\beta^A\beta^A$	$\beta^{Thal}\beta^A$	$\beta^E\beta^A$	$\beta^E\beta^E$	Total
(αα/αα)	253	6	40	11	310
$(-\alpha^{3.7}/\alpha\alpha)$	55	4	13	4	76
$(-\alpha^{4.2}/\alpha\alpha)$	3		1		4
$(\alpha^{CS}\alpha/\alpha\alpha)$	12		17	3	32
$(-\alpha^{3.7}/-\alpha^{3.7})$	13		4		17
$(-\alpha^{3.7}/-\alpha^{4.2})$			2		2
$(-\alpha^{3.7}/\alpha^{CS}\alpha)$	1		3	1	5
$(-\alpha^{4.2}/\alpha^{CS}\alpha)$	1				1
$(\alpha^{CS}\alpha/\alpha^{CS}\alpha)$	1				1
$(--^{SEA}/\alpha\alpha)$	172	2	1		175
$(--^{Tot}/\alpha\alpha)$	138	1	3		142
$(--^{SEA}/-\alpha^{3.7})$	10	1			11
$(--^{Tot}/-\alpha^{3.7})$	11				11
$(--^{SEA}/\alpha^{CS}\alpha)$	4				4
$(--^{SEA}/-\alpha^{4.2})$	1				1
$(--^{Tot}/-\alpha^{4.2})$					
$(--^{SEA}/--^{SEA})$	1				1
$(--^{Tot}/--^{SEA})$	4				4
Total	679	14	84	19	796

[a] This work was supported by U.S. Public Health Service MCH Grant MCJ-151002-01.

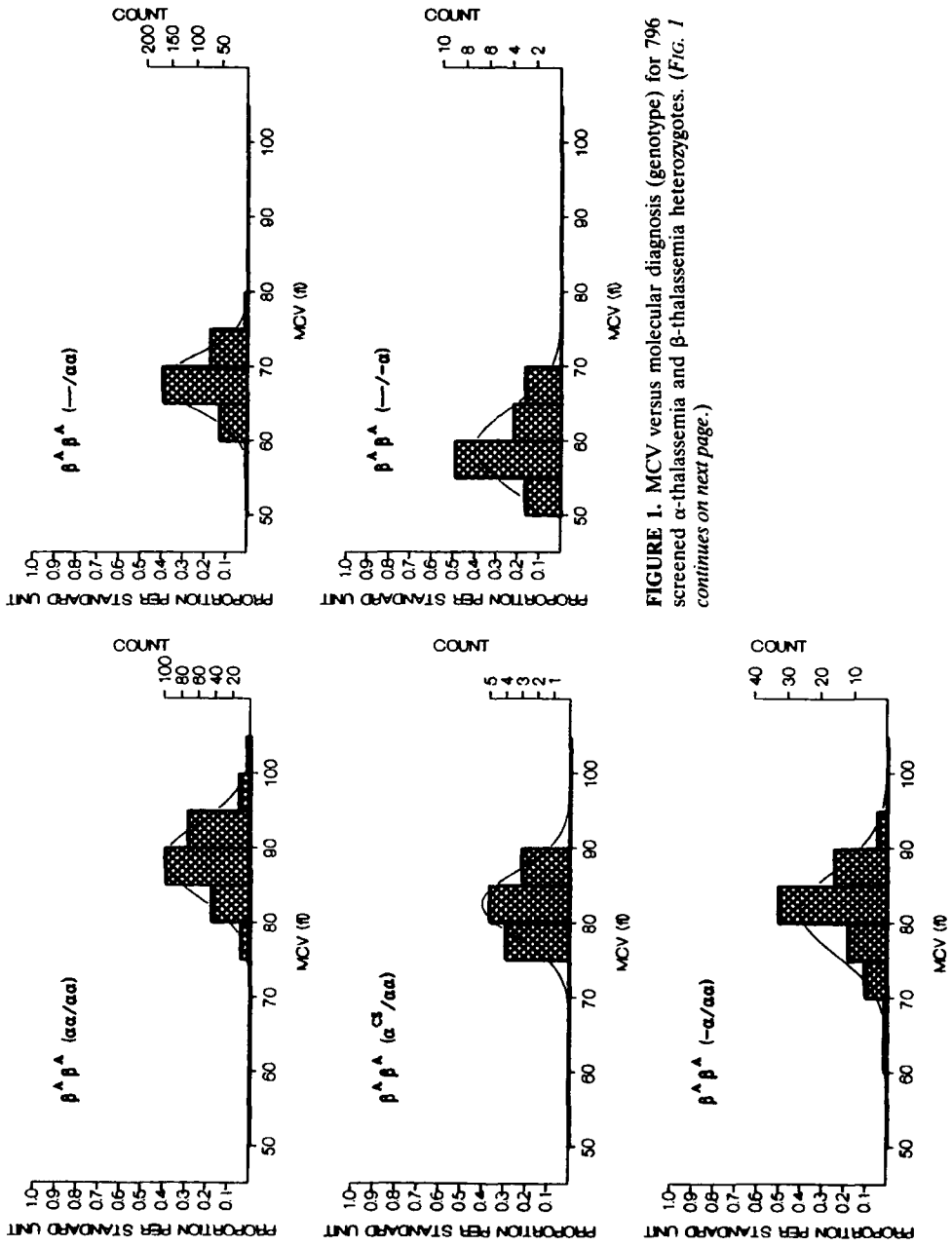

FIGURE 1. MCV versus molecular diagnosis (genotype) for 796 screened α-thalassemia and β-thalassemia heterozygotes. (*FIG. 1 continues on next page.*)

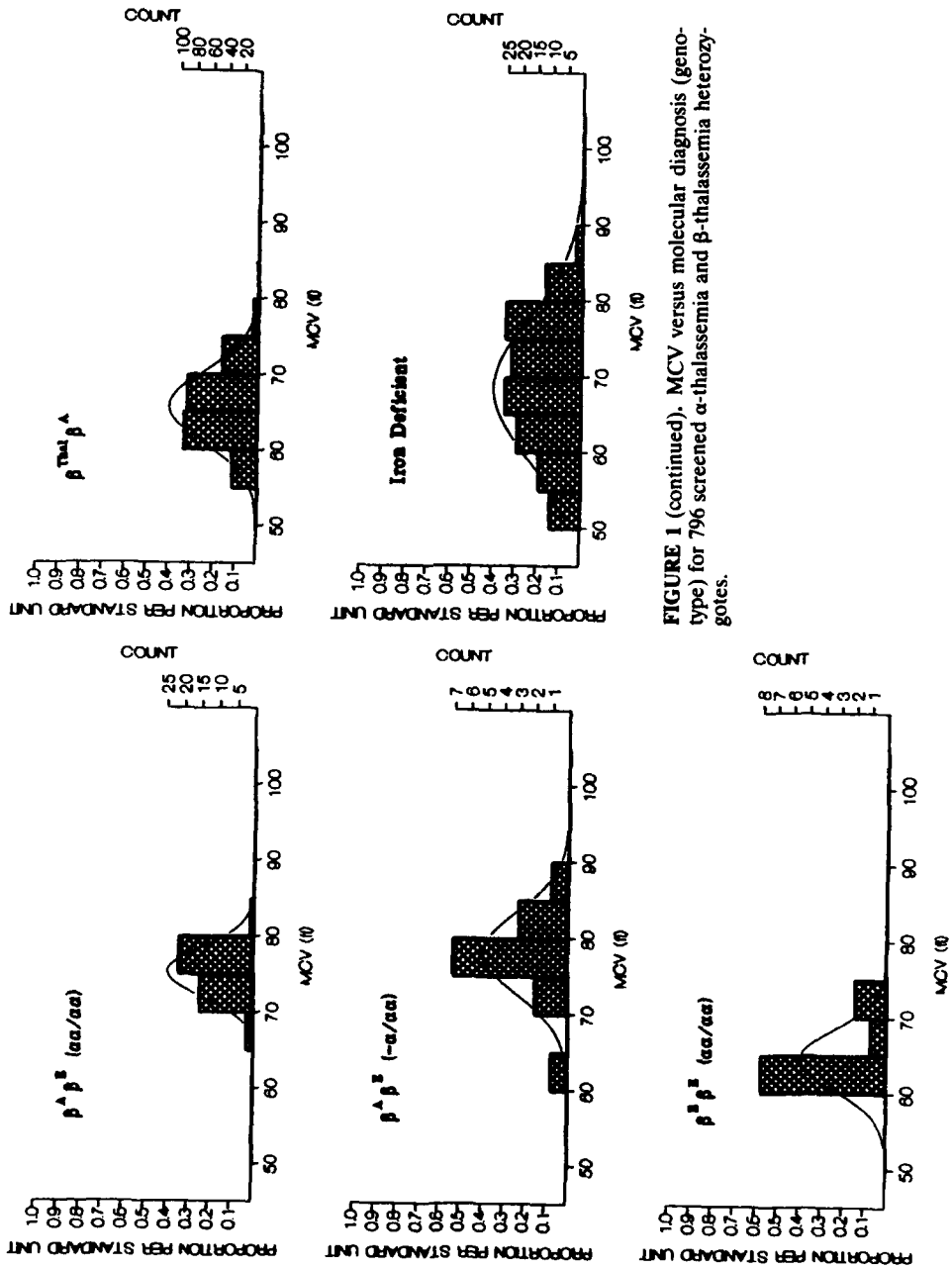

FIGURE 1 (continued). MCV versus molecular diagnosis (genotype) for 796 screened α-thalassemia and β-thalassemia heterozygotes.

variants. The α_1^{Thal}, Hb H, and β^{Thal} heterozygotes rarely overlapped the normal range. RDW[b] and ZPP[c] were not raised in α^{Thal} heterozygotes, slightly raised in β^{Thal} heterozygotes, but markedly raised in Hb H ($--/-\alpha$) and Hb H/Constant Spring ($--^{SEA}/\alpha^{CS}\alpha$) heterozygotes. A few β^{Thal} heterozygotes or Hb H disease subjects had marked anisocytosis (high RDW), raising MCV and MCH to the lower limits of normal. Measurement of zinc protoporphyrin was useful for ruling out iron deficiency, except that it was also raised by any reticulocytosis.

Hb Constant Spring, Hb E, Hb S, and single-deletion α_2^{Thal} heterozygotes overlapped both normals and other thalassemia heterozygotes. Simple β-globin heterozygotes were indistinguishable from mixed heterozygotes who also had α-globin variants.

In conclusion, red cell indices are very useful in screening for α-globin and β-globin variants, but they will not identify all variants; and they cannot distinguish the many variants from one another.

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[b] RDW: red cell volume distribution width, expressing the degree of anisocytosis.
[c] ZPP: red cell zinc protoporphyrin, raised with impaired or increased heme synthesis.

The Relevance of Mean Corpuscular Volume of Heterozygotes to Prenatal Diagnosis of β-Thalassemia[a]

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Prenatal diagnosis of β-thalassemia in Israel has recently shifted to direct detection of mutations in fetal DNA amplified by the polymerase chain reaction (PCR). This technique requires prior knowledge of each family's mutations. Israeli thalassemia heterozygotes come from diverse ethnic groups whose mutations have been incompletely characterized. Sixteen mutations have been identified to date in Israel.

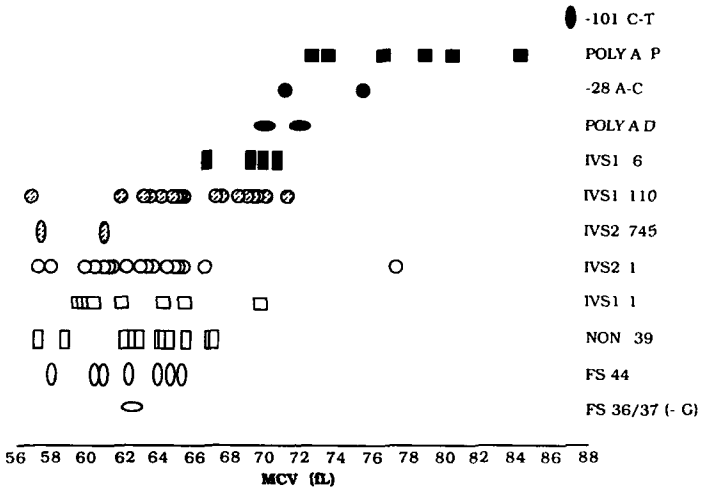

FIGURE 1. Relationship between the type of mutation and the MCV of heterozygotes. DNA isolated from 78 individuals was subjected to *in vitro* gene amplification using PCR and screened for various mutations using radiolabeled allele-specific oligonucleotide probes, as described.[4] Each point represents the MCV of one individual tested for each of the mutations listed at the right. *Open symbols,* β^0 mutations; *hatched symbols,* internal IVS (β^+) mutations; *closed symbols,* β^+ mutations.

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TABLE 1. β-Thalassemia Mutations in Israeli Ethnic Groups and Their Relationship to Mean Corpuscular Volume (MCV)

			Ethnic Group[a]				
Mutation	Type	Average MCV	Kurdish Jews	Medit. Jews	Moslem Arabs	Christ. Arabs	Druze
		Nonfunctional RNA					
Nonsense							
Codon 39 (C→T)	β^0	69.2	+	+	+		
Frameshift							
Codon 36/37 (−G)	β^0	62.3	+				
Codon 44 (−C)	β^0	62.3	+				
		RNA Processing Mutants					
Splice junction							
IVS-1 nt 1 (G→A)	β^0	62.8			+	+	
IVS-2 nt 1 (G→A)	β^0	63.3	+				+
Consensus changes							
IVS-1 nt 6 (T→C)	β^+	69.2	+		+		
Internal IVS changes							
IVS-1 nt 110 (G→A)	β^+	66	+	+	+		
IVS-2 nt 745 (C→G)	β^+	59.4		+			
		Promoter Mutations					
−28 A→C	β^+	73.6	+				
−101 C→T	β^+	87	+				
		Cleavage Mutations					
AATAAA→AATAAG	β^+	77.7	+				
AATAAA→A_____	β^+	71			+		

[a]Medit., Mediterranean; Christ., Christian.

Heterozygous β-thalassemia causes microcytosis,[1] but the mean corpuscular volume (MCV) can vary greatly. Previous studies[2-3] which showed the association between MCV and the type of mutation were done on carriers of similar ethnic background. We confirmed this correlation and its reliability even across ethnic boundaries. We therefore suggest that this universally available parameter, the MCV of heterozygote relatives, can be used to prioritize screening for multiple mutations in an ethnically complex population.

We studied 78 β-thalassemia heterozygotes over age ten from five ethnic groups (TABLE 1). MCV was measured (Coulter S Plus IV) and β-thalassemia mutations were identified as described.[4]

Twelve mutations were found (TABLE 1). Their distribution relative to MCV is seen in FIGURE 1. Forty-four individuals carried any of five β^0 mutations. The MCVs of 42 of those were in the range of 57.8–66.5 (average, 62.9). Thirty-four subjects carried any of seven β^+ mutations. Their MCVs were in the range of 57–87.3 (average, 69.1). Detailed analysis revealed that promoter, consensus or cleavage mutations resulted in higher MCVs (range, 66.6–87.3; average 74.2). A very low value for an MCV was observed with each of two internal IVS mutations: IVS-2 nt 745 and IVS-1 nt 110; the latter was associated with a wide range of MCV values (57–72).

We conclude that the MCV of related heterozygotes can be used to estimate which mutations are likely to be found in a given family. The major exception was IVS-1 nt 110. This information can be utilized to plan a strategic approach to identification of mutant alleles in a genetically heterogeneous population.

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Prenatal Diagnosis of β-Thalassemia in a Twin Pregnancy Using Allele-Specific Oligonucleotides

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First-trimester prenatal diagnosis of β-thalassemia using restriction fragment length polymorphism (RFLP) analysis on DNA prepared from chorionic villus samples (CVS) is now a well-established procedure. However, this technique is only applicable in about 80% of families at risk for the disease, because informative polymorphisms are not always found.

These problems can be largely overcome by direct detection of the mutation using allele-specific oligonucleotides (ASOs). The 5′ segment of the β-globin gene was amplified using 30 cycles of the polymerase chain reaction (PCR) in a Perkin Elmer/Cetus thermal cycler. Cycle conditions were 92 °C for 1 min, 55 °C for 1 min, and 72 °C for 1.5 min. The amplifying primers were GTA-CGG-CTG-TCA-TCA-CTT-AGA-CCT-CA and TTG-CCC-ATA-ACA-GCA-TCA-GGA-GTG-GA. Aliquots of denatured PCR product were applied to GeneScreen Plus in a dot blot apparatus. Detection oligonucleotides were 5′ labeled with T4 polynucleotide kinase and purified on 20% polyacrylamide gels. Duplicate filters were hybridized with wild-type and mutant probes. For detection of the codon 39 mutation, the wild-type probe was CCT-TGG-ACC-CAG-AGG-TTC-T, and the mutant probe was AGA-ACC-TCT-AGG-TCC-AAG-G. For the IVS-1 nt 110 mutation, the wild-type probe was CTG-CCT-ATT-GGT-CTA-TTT-T, and the mutant probe was CTG-CCT-ATT-AGT-CTA-TTT-T. The filters were prehybridized and hybridized at 42 °C in 5× SSPE, 0.5% SDS and 5× Denhardt's solution. They were washed twice with 6× SSC at room temperature for 10 min and twice with 50 m*M* Tris-HCl, pH 8.0, 2 m*M* EDTA, 0.1% SDS, and 3*M* tetramethyl-ammonium chloride (TMACL) at 61 °C for 15 min.

Hybridization with ASOs has been used for prenatal diagnosis in a twin pregnancy. A couple of Italian descent first presented for prenatal counseling in 1978. As there were no siblings or other family available for study, fetal blood sampling was carried out at 19 weeks; a homozygous fetus was diagnosed and the pregnancy was terminated. RFLP analysis on DNA from both parents and the fetus was only partially informative. Further tests using ASOs revealed that the father had an IVS-1 nt 110 mutation and the mother had a codon 39 mutation.

Early in 1989 the couple again presented for prenatal diagnosis. Ultrasound examination at 8 weeks revealed a twin pregnancy. Trophoblast tissue was obtained from each placenta and used for ASO and karyotype analysis. These studies (FIG. 1) revealed a male fetus with both the codon 39 and the IVS-1 nt 110 mutation (i.e., homozygous for β-thalassemia) and a female fetus with only the IVS-1 nt 110

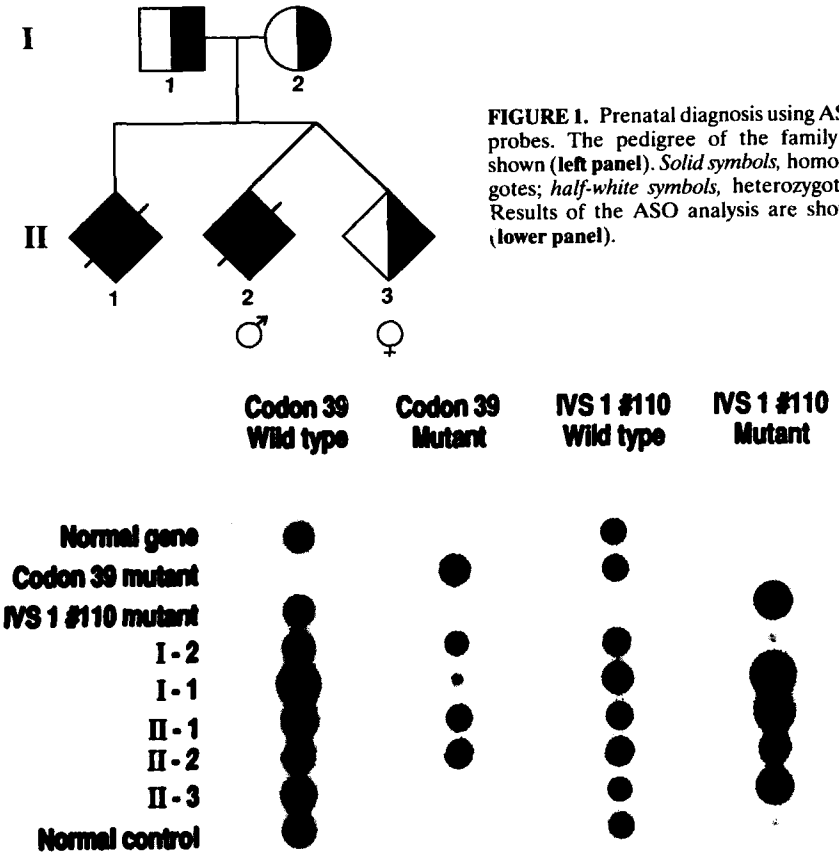

FIGURE 1. Prenatal diagnosis using ASO probes. The pedigree of the family is shown (**left panel**). *Solid symbols,* homozygotes; *half-white symbols,* heterozygotes. Results of the ASO analysis are shown (**lower panel**).

mutation (i.e., heterozygous). After further counseling, the couple elected for selective termination of the affected fetus, and this was performed at 15 weeks by intracardiac injection of potassium chloride. The pregnancy continued uneventfully, and a healthy female infant was delivered at 36 weeks. ASO analysis on the cord blood confirmed that the child had only the IVS-1 nt 110 mutation.

Selective termination of an affected fetus is an option for couples confronted with the problem of a prenatally diagnosed abnormality in one fetus of a twin pregnancy.

DNA Diagnosis of Thalassemias in Chinese by a Non-Radioactive Method[a]

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Current techniques for prenatal diagnosis of thalassemia mostly rely on radioactive probes, which are difficult to obtain in most developing countries. Here we report a simple and non-radioactive approach to the prenatal diagnosis of thalassemia syndrome in China, where this disorder is the most prevalent hemolytic anemia in the south. The polymerase chain reaction (PCR) procedure was used to amplify DNA directly from lysed villi or amniotic fluid cells and leukocytes according to a modification of the PCR procedure.[1,2] The α primer pair amplifies a 136-bp sequence of the α cluster region between ψα and α_2. The β primer pair directs the amplification of a 110-bp sequence from cap nucleotide (nt) 14 to codon 24 of the β-globin gene. For prenatal diagnosis of α-thalassemia, 10 μl of PCR product was separated on 2% agarose or 3% NuSieve agarose by gel electrophoresis, and the amount of the amplified α-globin DNA was directly visualized on the ethidium bromide–stained gels under ultraviolet light. For prenatal diagnosis of β-thalassemia, 10 μl of amplified β-globin DNA was digested with 10 U of *Hgi*A I, which has a polymorphic recognition site at codon 2. The polymorphism (110 bp; 65 + 45 bp) was analyzed on 3% NuSieve agarose by gel electrophoresis.

A couple from South China who already had a fetus with Hb Bart's hydrops fetalis requested prenatal diagnosis during the second pregnancy. Based on detailed hematologic and Hb examinations, we determined that both α- and β-thalassemia occurred in this family and that the parents both were double heterozygotes. The prenatal diagnostic result (FIG. 1) showed that the amount of the amplified α-globin DNA (136 bp) from the fetus was only half of that from the normal control. Analysis of the *Hgi*A I–digested amplified β-globin DNA revealed that only the 110 bp fragment appeared in the samples from the paternal grandmother and the maternal grandmother (both β-thalassemia minor); while three amplified fragments of 110/65 + 45 bp were found in the paternal grandfather (normal β-globin), maternal grandfather (normal β-globin), father (β-thalassemia minor), mother (β-thalassemia minor) and fetus. Thus, the fetus was antenatally diagnosed to carry both the α-thalassemia-1 and β-thalassemia genes. The result was fully confirmed by hematologic and DNA analysis of the cord blood sample.

So far, a total of 22 cases at risk for Hb Bart's hydrops fetalis and 30 fetuses at risk

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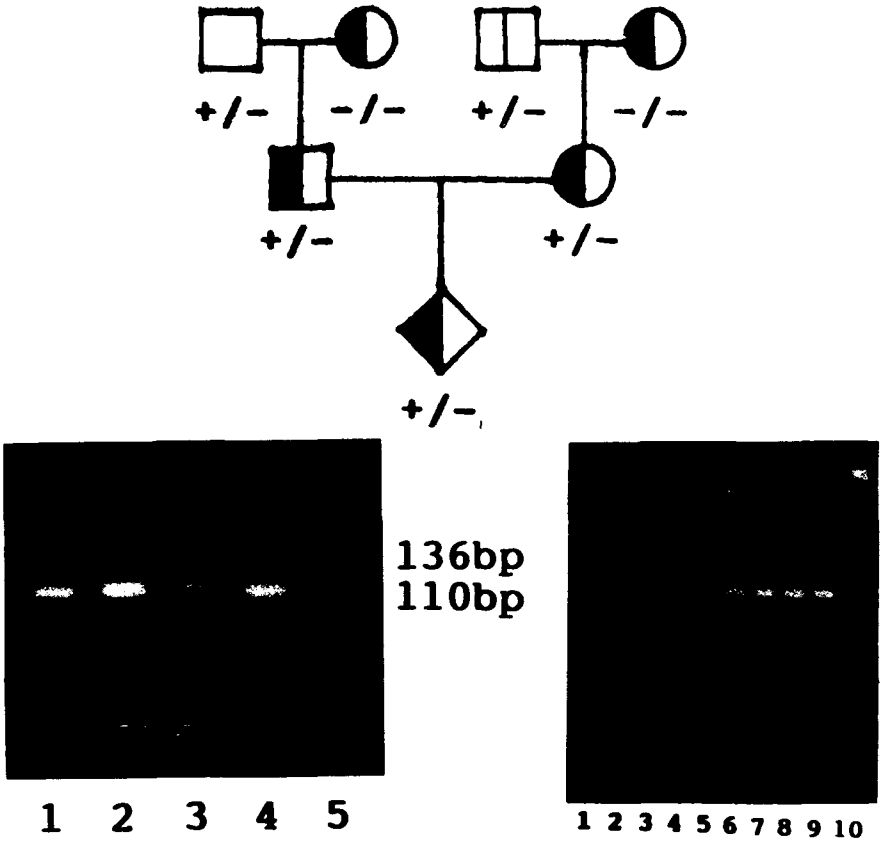

FIGURE 1. The prenatal diagnosis of thalassemias through an analysis of amplified DNA on 3% NuSieve agarose by gel electrophoresis (TEB buffer, pH 8.6, 40 mA, 0.5 h). **(Upper panel)** Pedigree of family analyzed. **(Lower left panel)** Detection of an α-globin gene deletion. The amplified α-globin DNA is 136 bp in length; a 110-bp segment of amplified β-globin DNA is present as an internal control. **(Lane 1)** Hb Bart's hydrops fetalis (positive control), **(lane 2)** mother, **(lane 3)** chorionic villi sample from fetus, **(lane 4)** father, **(lane 5)** normal control. **(Lower right panel)** Prenatal diagnosis of β-thalassemia by analysis of *Hgi*A I polymorphisms in amplified DNA. **(Lanes 1 and 9)** Normal control, **(lane 2)** paternal grandfather, **(lane 3)** father, **(lane 4)** paternal grandmother, **(lane 5)** chorionic villi sample from fetus, **(lane 6)** maternal grandfather, **(lane 7)** mother, **(lane 8)** maternal grandmother, **(lane 10)** 123-bp ladder DNA markers.

for β-thalassemia major have been antenatally diagnosed by use of this method. The total procedure for the DNA analysis required only 5–6 h.

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Prenatal Diagnosis of Thalassemia Intermedia: Is It Justified?

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The indications for prenatal diagnosis of thalassemia intermedia are still vaguely defined and, in many instances, the question arises whether molecular techniques would allow couples at risk to make more informed decisions. To obtain a more concrete opinion on this matter we have collected the clinical and laboratory data of a large number of patients with thalassemia intermedia and tabulated them according to the underlying molecular defects. In this communication, thalassemia intermedia denotes a double-dose β-thalassemia where the patients grow well, do not develop gross bone deformities, have a relatively good quality of life, and do not need regular transfusions.

The survey includes 46 adult patients with thalassemia intermedia meeting the criteria defined above. Hematological and biochemical values were determined by standard methods. Molecular studies consisted of the identification of (a) the underlying β-gene cluster standard haplotypes by restriction fragment length polymorphism analysis (RFLP), (b) the molecular defect by the polymerase chain reaction (PCR) with specific oligonucleotides, (c) the (occasionally) interacting δβ- or α-thalassemia, and the −158 *Xmn* I site 5′ to the $^{G}\gamma$ genes.

The results are summarized in TABLES 1 and 2. Thalassemia intermedia displays a large heterogeneity with regards to hematological values, biochemical parameters, and underlying mutations. In this study, all the patients were relatively well, but none was virtually problem-free. Problems included mild bone deformities, leg ulcers, easy fatigue, and cholelithiasis. Moreover, patients had a limited capacity and reported frequent absence from work. Most of them had been splenectomized.

When a large number of medical and paramedical staff members was asked whether they would accept having children with these types of thalassemia intermedia, the unanimous answer was negative. The conclusion is that prenatal diagnosis cannot be denied selectively to couples at risk for thalassemia intermedia.

In most cases, the observed phenotypes were in keeping with the underlying molecular lesions (TABLE 2). In detail, carriers of two mild thalassemia genes (β^{++}/β^{++}), compound β^{++}/β^{+} heterozygotes, and homozygous β^{+}/β^{+} patients displayed progressively decreasing levels of Hb A and β mean corpuscular hemoglobin (β-MCH) values; the calculated level of β chain synthesis per gene or cell was consistent with the reported (or expected) inhibition of β mRNA transcription and processing. In the patients who have an inherent capacity to produce high levels of γ

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TABLE 1. Frequency of the β Gene Cluster Haplotypes and of the β-Thalassemic Mutations in Greece

		Normal Chromosomes (%)	Chromosomes of Patients: Thalassemia Major (%)	Chromosomes of Patients: Thalassemia Intermedia (%)
Haplotype[a]				
I		38.3	47.9	36.8
II		15.8	17.5	5.2
III		2.8	2.8	7.0
IV		1.9	0.9	—
V		12.3	12.0	5.2
VI		2.2	7.4	33.3
VII		4.4	4.6	7.0
VIII		—	—	—
IX		19.3	4.1	5.5
Other		3.0	2.8	—
Thalassemic mutation[b]				
IVS-1 nt 1	β^0		16.1	4.7
IVS-2 nt 1	β^0		2.3	3.5
FS codon 5	β^0		—	1.2
FS codon 6	β^0		2.3	1.2
FS codon 8	β^0		0.4	—
β^0 −39	β^0		19.4	5.8
IVS-1 nt 5	β^+		—	—
IVS-1 nt 110	β^+		42.0	30.2
IVS-2 nt 745	β^+		1.4	4.7
β −87	β^{++}		2.3	5.8
IVS-1 nt 6	β^{++}		11.5	33.7
β −101	β^{++}		—	1.2
Unknown			2.3	8.1

[a] n = 316 for normal chromosomes, 217 for patients with thalassemia major, and 57 for those with thalassemia intermedia.

[b] n = 217 for chromosomes of patients with thalassemia major, and 86 for those with thalassemia intermedia. FS, frameshift.

TABLE 2. Thalassemia Intermedia Phenotypes in Relation to Genotypes: Main Groups

Mutations	Mean Hematological and Biochemical Values: Hb A (%)	Hb F (%)	Hb (g/dl)	MCH (pg)	β-MCH (pg)	% of Normal	α Genes (n)
β^{++}/β^{++} (n = 6)	86.4	9.9	8.1	20.1	8.7	58.0	4
β^{++}/β^{+} (n = 5)	57.5	39.2	7.8	22.6	6.5	43.4	4
β^{++}/β^{0} (n = 4)	37.2	60.5	9.3	24.4	4.5	29.9	4
β^{+}/β^{+}							
3 Patients	31.0	65.0	7.6	25.0	4.0	26.4	4
2 Patients	90.0	9.0	8.5	25.4			4
β^{0}/β^{0} or $\beta^{0}/\delta\beta$-thalassemia (n = 4)	0	98.0	10.1	26.9			4

chains, the MCH becomes higher, while the calculated level of β chain synthesis per gene or cell decreases proportionally and, occasionally, more than expected. Three β^0/β^0-thalassemia patients had high hemoglobin levels and almost 100% Hb F. One of them was −/− for the −158 *Xmn* I polymorphism; the mechanism of high Hb production in the other two remains undefined.

Exceptions from this general pattern were not rare. Understanding these requires additional studies directed to (a) identification of yet unknown mutations (8.2%), (b) identification of factors promoting γ-chain synthesis, and (c) searching for the simultaneous presence of non-deletional types of α-thalassemia which are known to occur in this country.

Other interesting but rare combinations leading to thalassemia intermedia among our patients were the interaction of β-thalassemia with β-Knossos and β-Koln, and the simultaneous presence of one β-thalassemia gene along with three + two α-globin chain genes.

Feasibility of Routine Prenatal Screening for β-Thalassemia Trait

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We have investigated the feasibility of screening for β-thalassemia trait within a prenatal hemoglobinopathy screening program.[1-3] We invited the participation of all prenatal care providers in the Rochester, New York, region; currently we are receiving blood drawn for other purposes at the first prenatal visit from 71% of the pregnancies in the metropolitan area. Of 39,253 pregnancies screened in 92 months, 264 (1 in 149) occurred in women with β-thalassemia trait, consisting of 126 Italians, 80 blacks, 48 other Europeans, 5 Vietnamese, 2 Hispanics, 2 East Indians, and 1 Lao. With their providers' permission, we notified these women of their positive results by telephone or certified letter and offered them counseling. Of these 264, 196 (74%) came for counseling.

The women counseled had an average age of 24.6 ± 5.7 years, an average of 12.3 ± 2.3 years of education, and an average of 0.63 children. The majority of the counselees were married (55%), were living with the father of the fetus (65%), were employed (53%), were previously unaware of their trait status (71%) and unaware of the risk to the offspring of a person with β-thalassemia trait (84%), had not planned their pregnancy (51%) but now wanted it (98%), and were early enough in gestation for prenatal diagnosis if indicated (87% before 18 weeks at time of phlebotomy). The counselees generally understood the information provided and thought their detection appropriate even though most providers had not asked for consent to screen.

Of these 163 counselees, 110 (67%) had their partners tested. Of these 110, 7 partners (6.4%) also had a hemoglobinopathy. Of the 6 offered prenatal diagnosis, 4 chose amniocentesis; the disease genotype was demonstrated or excluded in each case. We conclude that receptivity to thalassemia screening in this prenatal population is high; 74% of those offered counseling received it, 67% of counseled women had their partner tested, and 67% of couples offered prenatal diagnosis because the partner was also positive had amniocentesis. Prenatal screening for β-thalassemia trait is feasible in the context of a prenatal screening program for detecting more common hemoglobinopathies.

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Newborn Screening for α-Thalassemia

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In May 1987 the Iowa Newborn Metabolic Screening Program began to screen newborns for sickle cell disease and related hemoglobinopathies. In February of 1988 the program became statewide.[1] Isoelectric focusing electrophoresis (IEF) of hemoglobin (Hb) was selected as the method for screening. IEF is able to distinguish over 100 variant Hbs, including the fast-moving Hb H and Hb Bart's.[2] The resolution of these Hbs offers the potential for screening newborns for individuals affected by α-thalassemia variants.

Blood samples were obtained on filter paper by heel puncture in newborn babies three or less days old. For confirmatory testing, EDTA-anticoagulated blood was obtained by venipuncture. Eluted hemolysates from filter papers were analyzed by IEF.[2] Hemolysates from the EDTA-containing samples were analyzed by high pressure liquid chromatography.[3] Once Bart's Hb was identified in a filter paper sample, confirmatory testing was requested on an EDTA-containing sample as soon as possible. The primary physicians of the newborn babies in whom Bart's Hb was detected were advised of that finding. Clinical follow-up and genetic counseling were recommended.

Of 65,049 babies screened in 22 months, 144 newborns were identified to have Bart's Hb (TABLE 1). One hundred and four of these babies had a confirmatory test done. The presence of Bart's Hb was confirmed in 96 babies; eight were normal. Forty newborn babies did not have a confirmatory test done. Two of these infants died before testing could be done. According to their Iowa State Birth Registry, 47 of the 144 babies were Asian (33%), 44 were black Americans (30%), 26 were white (18%), and in 27 the race was not known (19%) (TABLE 2). Nine families received genetic counseling. During the same interval of time, 392 infants with sickle cell hemoglobin were detected. This number includes both sickle cell trait and sickle cell disease.

In summary and conclusion, during a 22-month span, 144 newborns with Hb Bart's were detected by the newborn Hb screening program. This represented 37% of the number of newborns detected with sickle Hb during the same period of time. Thirty-three percent of the newborns with Bart's Hb were Asian and 18% were

TABLE 1. Results of Newborn Hemoglobinopathy Screening

	Initial Screen Bart's Hb		Confirming Screen			
			Bart's Hb		Normal	Not Done
Total Screened (*n*)	*n*	%[a]	*n*	%[a]	(*n*)	(*n*)
65,049	144	0.22	96	0.15	8	40[b]

[a]%, % of total newborns screened.
[b]Two babies died before testing could be done.

TABLE 2. Race of Newborns with Bart's Hb

Race	Initial Screen Bart's Hb	Confirming Screen Bart's Hb
Asian	47	38
Black	44	29
White	26	17
Unknown	27	10

white. These are the two groups likely to be at risk for hematologically significant α-thalassemia disorders. Genetic counseling was given to only nine families. Bart's Hb is unstable and is degraded in the filter paper samples. The number of cases detected in our program thus represents only a minimum of the affected individuals. To detect a greater proportion of affected individuals, an anticoagulated blood sample would be required. To date there is no evidence to suggest that diagnosis of clinically significant α-thalassemia at birth will improve the mortality or morbidity of the individuals affected with the disease. Furthermore, genetic counseling, which may affect, in theory, the incidence of serious α-thalassemia disorders, was not successful in our population. Although our program has documented that IEF of Hb from dry-blood specimens on filter paper can detect Bart's Hb, the application of this finding is unclear. We conclude that screening for α-thalassemia is feasible. The appropriate technique for screening has not been determined.

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Importance of House Calls in Detecting Heterozygotes in Families with Thalassemia

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The problem of thalassemia major is still important in Sicily, even if the birth rate of affected subjects has decreased in the last few years. We report the results of a new and more efficient strategy for detection of heterozygotes in order to give them appropriate information about the risk of their condition.[1,2]

We traced 260 first- and second-degree relatives of 7 children with thalassemia major treated at the Thalassemia Center of Catania University. A letter was sent to 183 of these subjects informing them about thalassemia and inviting them to contact us by telephone and undergo a hematological test to detect the carrier state. We received no answer; so, after 6 months, the social assistant went to 70 of these individuals and gave them the questionnaire shown in TABLE 1. Only the people who had never undergone blood tests were asked the last two questions shown in TABLE 1.

The number of affirmative answers to each of the first group of questions is reported in TABLE 1. None of the subjects had undergone the screening before having received the letter. Fifty-two out of 70 subjects had received the letter, and only 4 of them had come to the Thalassemia Center for screening after receiving the letter. Most of the interviewed subjects (84%) knew of the existence and distribution of thalassemia, and 79% of them knew that it is possible to detect heterozygotes by a simple hematological test. Moreover, 74% of the subjects knew they had some cases

TABLE 1. Questionnaire Given to Relatives at Risk for Thalassemia

Question	Affirmative Answers	
	Ratio	%
Did you receive our letter?	52/70	74
Did you already know about the existence and distribution of thalassemia?	59/70	84
Did you know there is a simple diagnostic test for detecting heterozygotes?	47/59	79
Did you know that there are some cases of thalassemia in your family?	52/70	74
Did you know you were in a high-risk condition?	45/52	86
Did you undergo the screening test—		
Before the letter?	0/70	100
After the letter?	4/52	7.6
Why did you not undergo the screening test?	See text	
What does your relative's disease mean for you?	See text	

in their family, and 86% of these knew they were in a high-risk condition. The answers to the last two questions showed that all the subjects who did not undergo the screening justified themselves with an excuse such as "I had no time" and considered their relative's disease as an unavoidable "calamity" which must be ignored.

After the house calls all the individuals contacted came to the Thalassemia Center for screening.

It seems that information through the mail has very little effect, and the answers to the questionnarie show that there is a psychological resistance to the high-risk group. Although the number of people who undergo thalassemia screening has increased tenfold in the last ten years as the result of better and more widely distributed information, the subjects at high risk are indifferent to the thalassemia problem. They consider the disease of their relative a calamity and prefer to ignore it. Only direct contact during house calls can remove the resistance. This efficient procedure can be performed in any town where the social assistant can link the thalassemia center and relatives at high risk.

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Impact of a Thalassemia Screening Program on the Attitudes of a Multi-Ethnic Population[a]

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The attitudes of 1628 adult participants over age 18 were surveyed in a thalassemia screening project that has offered statewide carrier testing, genetic counseling, and prenatal diagnosis to high-risk Southeast Asian groups in Hawaii since May 1985.[1] Screening objectives included detecting heterozygotes[2] to reduce the occurrence of thalassemia major,[3] providing genetic counseling in the context of individual cultures,[4,5] as well as evaluating cultural and religious attitudes toward screening and prenatal diagnosis.[6,7] We found that psychosocial, cultural, and religious attitudes have become modified by the project.

Initial opposition to the project arose with rumors of blood-selling and with beliefs that the spirit was lost by blood-drawing, that iron was a cure-all, and that only newly arrived immigrants had anemias.

Among the surveyed, 688 were male (539 married) and 940 were female (685 married); 298 were Laotion, 737 Filipino or part-Filipino, 393 Chinese or part-Chinese, 200 of other races; 27% had α^{Thal} variants, 7% had Hb E, and 9% had β^{Thal} variants.

TABLE 1. Participant Attitudes Toward Thalassemia Screening and Prenatal Diagnosis

Before Screening	After Screening
Beliefs	
Families closed and private	Families more open to society
Acceptance of folk medicine	Acceptance of Western medicine
Fear of permanent weakness from blood loss	Acceptance of blood-drawing
Reproduction	
Large unplanned families	Smaller planned families
No contraception	Acceptance of contraception
No amniocentesis	Acceptance of amniocentesis
No abortion	Acceptance of abortion
Health Behavior	
Limited health education	Improved health clinic attendance
Resistance to social services	Acceptance of social services
Belief only immigrants needed test	Acceptance of testing for residents
Directive health care	Non-directive health care
Non-participation by ethnic/religious organizations	Supportive participation by ethnic/religious organizations

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After initial education, counseling and testing, structured questionnaires were used to assess the subjective attitides of participants toward screening and prenatal diagnosis. Participant attitudes before and after screening are summarized in TABLE 1. If found to be heterozygous, 37% of the participants reported after screening that they would feel bad, 34% would not, 29% were indifferent; 70% would recommend screening to relatives (see FIG. 1); 68% would have prenatal diagnosis; 40% would abort a fetus with thalassen.ia major. The Filipinos, almost all of Roman Catholic faith, were more reluctant to abort than were the other races.

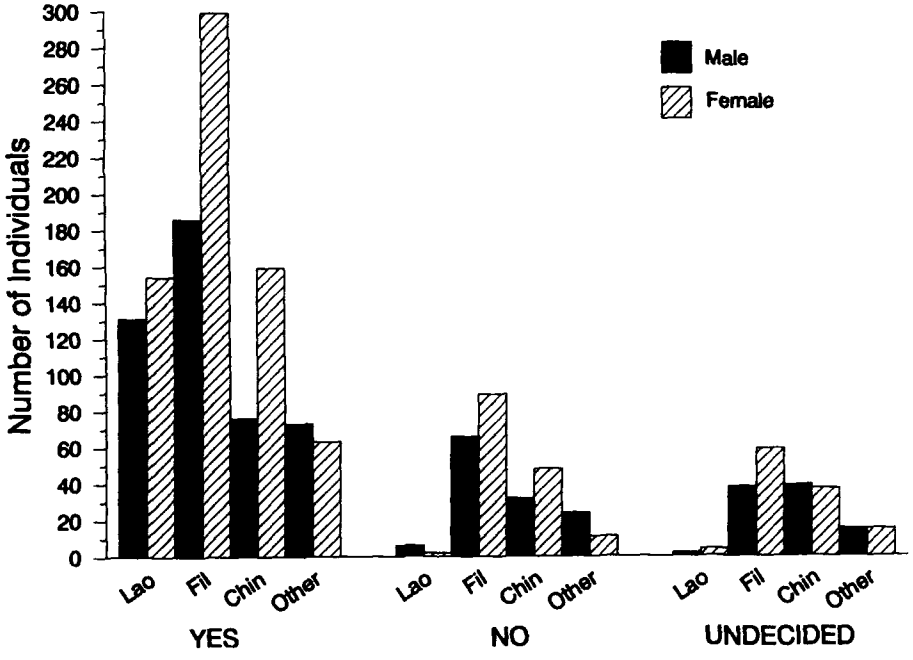

FIGURE 1. Attitudes of participants in screening project to recommending screening for thalassemia to their relatives. Fil, Filipino or part-Filipino; Chin, Chinese or part-Chinese.

Credibility in the screening project was generated by the cooperation of physicians, the enthusiastic support of key ethnic and religious organizations, and the evidence that some families in the community had been helped. Some of those screened have asked for blood transfusions after they saw how this had benefited those who were detected to have a thalassemia major.

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Successful Pregnancy following Resolution of Secondary Amenorrhea due to Hemochromatosis in β-Thalassemia Intermedia

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Delayed sexual development and infertility are common in women with homozygous β-thalassemia. Anovulation is thought to be due to hypogonadotrophic hypogonadism caused by deposition of hemosiderin in the hypothalamus and pituitary. A recent review[1] was able to identify only 15 known pregnancies with 12 live births in 9 women with homozygous β-thalassemia. Three of these women had β-thalassemia major and 6 had β-thalassemia intermedia.

Iron chelation therapy can reduce the iron load in patients with hemochromatosis. Unfortunately, end-organ damage is generally irreversible.

We report what we believe to be the first successful pregnancy in a woman with homozygous β-thalassemia and hemochromatosis following reversal of secondary amenorrhea with iron chelation therapy.

A 30-year-old married nulligravida woman with non–transfusion-dependent β-thalassemia intermedia, splenectomized at age 7, presented in August 1985 with a 6-month history of secondary amenorrhea. Menarche was at age 13 and menses had previously been regular. Exam revealed frontal bossing, dental malocclusion, scleral icterus and bronzed skin. Laboratory results were hemoglobin (Hb) 7.6 g/dl; hematocrit 23.8%; Hb electrophoresis: (A) 59.5%, (A_2) 4.5%, (F) 36%; Hb F homogeneously distributed; serum ferritin 1945 ng/ml; serum Fe 174 mcg/dl and total iron-binding capacity (TIBC) 192 mcg/dl; hepatic Fe by magnetic susceptometry 14×10^3 mcg/dl (normal is < 250). Thyroid function tests were normal. Subcutaneous desferrioxamine, 2 g nightly, was begun in November 1985. Menses resumed in November 1987. Ferritin at that time was 16.9 ng/ml, so treatment frequency was reduced by half. In February 1989, she reported that her last menstrual period had been in November 1988. Hb at that time was 7 g/dl and ferritin 66 ng/ml. The pregnancy test was positive. Desferrioxamine was discontinued. She was transfused with leukocyte-depleted packed red blood cells to maintain her Hb at greater than 9 g/dl, requiring 18 units overall. A healthy daughter was born at term. Cesarean section was performed for obstetrical indications. One month post partum, ferritin was 963 ng/ml, serum Fe 46 mcg/dl, and TIBC 191 mcg/dl (FIG. 1).

Successful pregnancy can be achieved in women with homozygous β-thalassemia following reversal with aggressive chelation therapy of secondary amenorrhea due to hemochromatosis.

Transfusional support to correct severe maternal anemia may help maintain normal fetal growth by permitting adequate placental oxygen exchange. It may also prevent the development of maternal high-output congestive heart failure.

Of note, our patient received desferrioxamine therapy prior to the realization

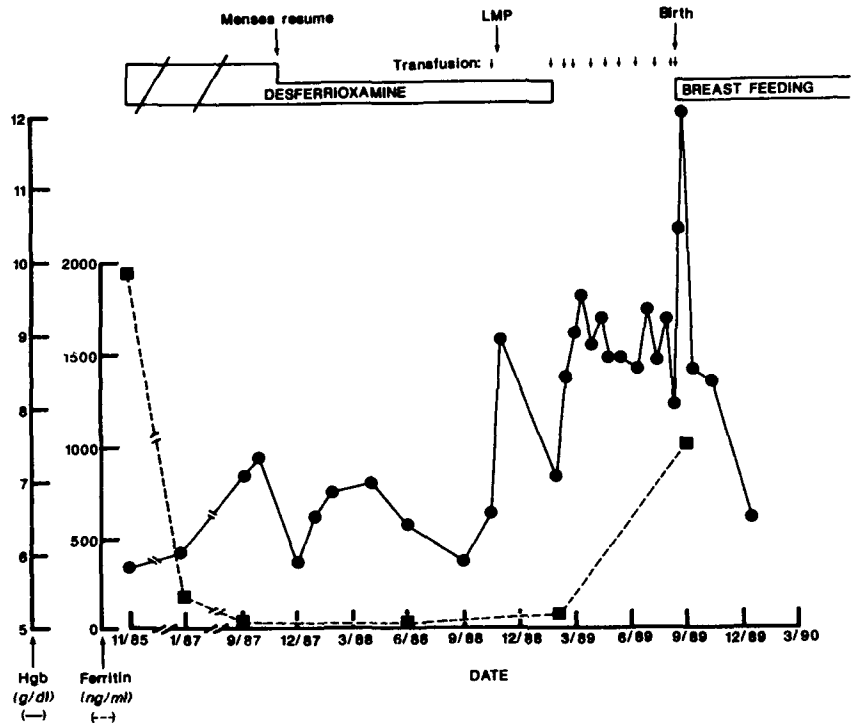

FIGURE 1. Clinical course of a woman with homozygous β-thalassemia and hemochromatosis who had a successful pregnancy after reversal of her secondary amenorrhea with iron chelation therapy.

that she was pregnant. She self-administered the drug for 14 consecutive days during weeks 3 and 4, and 10 consecutive days during weeks 7 and 8 of the pregnancy. No adverse fetal effects are apparent. Three other women[2-4] are known to have received desferrioxamine during pregnancy (to 16, 19 and 32 weeks, respectively) with the only complication of pregnancy being premature delivery in all three instances. The safety of desferrioxamine during pregnancy remains to be established.

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Serum Erythropoietin Levels in β-Thalassemia

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So far there are scanty reports concerning the behavior of serum erythropoietin (sEpo) levels in the disease thalassemia. We here report the results obtained by a preliminary study carried out on 32 subjects of both sexes, aged 5 to 50 years, all affected by β^+-thalassemia: 11 heterozygous, 16 homozygous with an intermediate clinical picture, and five homozygous with a clinical picture of thalassemia major and a regular blood transfusion regimen. Ten healthy subjects of both sexes and comparable in ages to the thalassemic subjects were controls. The diagnosis of β^+-thalassemia was made on the basis of family investigation and conventional hemometric laboratory parameters. The sEpo was measured by an ELISA method. In each group we compared the sEpo levels with the following hemometric parameters: hemoglobin level (Hb), hematocrit value (Hct), and fetal hemoglobin (Hb F).

The results of these studies are summarized in TABLE 1. In the heterozygous group, the median Hb and Hct values were 11.8 g/dl and 36.8%, respectively. The sEpo levels ranged from 45 to 108, with a median value of 75.6 mU/ml. In the thalassemia intermedia group, the Hb, Hct, and Hb F median values were 8.2 g/dl, 26.0%, and 35.1%, respectively. The sEpo levels ranged from 10 to 77, with a median value of 43.1 mU/ml. In the thalassemia major group, the median values of Hb, Hct, and Hb F were 10.3 g/dl, 36.7%, and 2.1%, respectively. The sEpo levels ranged from 33 to 72, with a median value of 47.2 mU/ml. In the control group, the median values of Hb and Hct were 14.4 g/dl and 43.6%, respectively. The sEpo levels ranged from 22 to 36, with median value of 27.1 mU/ml.

Our results show that in all thalassemic subjects, both homozygous and heterozygous, the sEpo levels are higher than in the controls ($p < 0.001$) and that the highest sEpo levels are found in the heterozygous group. Also, when the sEpo levels were compared to the hemometric parameters Hb, Hct and Hb F, a different behavior was observed among the groups. In the heterozygous group, for Hb and Hct an inverse correlation with sEpo levels was seen ($p < 0.001$). In the intermedia-major group, in which the Hb F level was accounted for, no correlation was found. This behavior of sEpo is difficult to explain. Usually, the circulating Epo levels are dependent on the kidney production and clearance rate, on inhibitors, and on erythropoietic bone marrow activity (utilization).[1,2] We believe that in all of our thalassemic subjects, especially in the homozygous ones, in which erythroid marrow hyperplasia is expected, the higher circulating Epo level may be due to its lesser utilization for the purpose of bone marrow erythropoiesis (perhaps because of changes in the affinity of Epo receptors).[3] In heterozygotes, an expansion of the mass of the bone marrow erythroid progenitor population might be expected to be minor compared to that in homozygotes, and an additional mechanism responsible for the high circulating Epo titers might also occur. Indeed, in these subjects it might be thought that, compared

TABLE 1. Comparison of Hemometric Values and Serum Erythropoietin Levels in Thalassemics

Parameter	Median Values ± SD[a]			
	Th. Het.	Th. Int.	Th. Maj.	Controls
Hb (g/dl)	11.8 ± 0.8	8.2 ± 1.0	10.3 ± 0.4	14.4 ± 0.8
Hct (%)	36.8 ± 1.2	26.0 ± 2.1	36.7 ± 1.2	43.6 ± 2.2
Epo (mU/ml)	75.6 ± 23.1	43.1 ± 16.1	47.2 ± 20.1	27.1 ± 4.7

[a]Th, Thalassemia; Het., heterozygotes; Int., intermedia; Maj., major.

to homozygotes, renal involvement in systemic organ damage by iron overload would be minor, so that the organ would still retain enough capability in the endogenous erythropoietin response. However, the pathophysiological significance of the changes of the sEpo levels in thalassemia syndromes remains to be established.

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Characteristics of Erythroid Progenitor Cells in β-Thalassemia Major and β-Thalassemia Intermedia

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The purpose of the studies presented here was to characterize β-thalassemia major (TM) and β-thalassemia intermedia (TI) erythroid progenitor cells (BFU-E) and to compare them with fetal (FB), newborn (NB), normal adult (AA), and sickle cell (SS) BFU-E.[1]

Mononuclear cells were obtained by Ficoll-hypaque centrifugation of peripheral blood and were cultured in methyl cellulose with erythropoietin for 21 days, as previously described.[2]

Data are presented in TABLE 1. No colonies were observed without the addition of erythropoietin. TM, TI, and AA bursts required similar doses of erythropoietin to achieve half-maximal growth (0.62, 0.46, and 0.74 units/ml, respectively). FB, NB, and SS bursts tended to be more sensitive to erythropoietin, requiring lower doses to achieve half-maximal growth (0.33, 0.33, and 0.31, units/ml, respectively).

In all TI (6/6), and most TM (9/10), NB (7/9), and AA (30/34) cultures, maximal growth occurred by day 16. In contrast, maximal growth was earlier in FB cultures (6/10 by day 13) and later in SS cultures (8/22 required 20 days). The mean day of maximal colony growth for each type of culture is shown in TABLE 1.

TM, TI, and SS patients often have white blood cell counts which are higher than those in normal individuals. As a result, the number of mononuclear cells/ml of blood obtained by Ficoll-hypaque centrifugation is higher in blood from hemoglobinopathies than in normal blood. Therefore, growth of BFU-E is reported as the number of colonies/ml of blood in an attempt to provide a physiologic comparison. On day 13, FB and NB colonies were similar in number and were the most numerous (TABLE 1). The colony numbers for TM, TI, and SS were similar to each other ($p > 0.05$) and significantly more numerous than the number of AA colonies (TM vs AA, $p = 0.0006$; TI vs AA, $p = 0.003$; SS vs AA, $p = 0.024$).

When the *maximal* values for colonies/ml were compared, differences in growth potential of the various types of erythroid progenitors were most apparent. In all categories, the mean number of colonies/ml increased compared with the values on day 13. FB and NB colonies were similar in number and the most numerous. TM, TI, and SS colonies were similar in number and were all significantly more numerous than AA colonies ($p < 0.01$ for AA vs TM, TI, and SS). Hemin increased colony numbers in all cultures, with maximal effect at 100 μ*M* in 14/17 AA, 8/10 TM and 4/4 TI and at 200 μ*M* in 7/10 SS cultures.

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TABLE 1. Characteristics of Erythroid Progenitor Cells (BFU-E)

Parameter	FB	NB	AA	TM	TI	SS
Erythropoietin, half-maximal dose	0.33	0.33	0.74	0.62	0.46	0.31
Mean day of peak growth	13.8	15.9	15.0	15.8	15.5	17.2
Mean colony/ml						
Day 13	2354	2384	271	672	852	426
Peak day	4089	3779	336	843	1160	917
Hemin response	+	+	+	+	+	+

Thus, TM, TI, and AA BFU-E had similar erythropoietin requirements and were less sensitive to erythropoietin than were FB, NB, or SS colonies. Maximal growth was by day 16 in TM, TI, and AA cultures but was earlier in FB and later in SS cultures. TM, TI, and SS BFU-E were similar in number and more numerous than AA BFU-E. FB and NB BFU-E were the most numerous. We conclude that TM and TI BFU-E are similar to each other but different from FB, NB, AA, and SS BFU-E.

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Immunodeficiency of β-Thalassemic Mice[a,b]

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There is some evidence that humans affected by β-thalassemia, a genetic anemia characterized by reduced production of β-globin, increased erythropoiesis, aberrant iron metabolism, and iron overloading, are immunodeficient and have perturbed ratios of lymphocyte subsets.[1] In such studies the relationship between iron metabolism and immunodeficiency remains uncertain because most thalassemia patients have undergone splenectomy and have received many blood transfusions. We have described a mouse model of β-thalassemia[2,3] in which the mouse survives without blood transfusion, has higher levels of serum iron and ferritin, and exhibits elevated iron storage in the spleen, liver, and kidney,[4] which makes it a good experimental system in which to examine the relationship between aberrant iron metabolism and immune dysfunction. This mouse model was used to compare the immume competence and to investigate the splenic lymphocyte populations of normal and β-thalassemic mice.

A hemolytic plaque assay was used to measure antibody-forming cell (plaque-forming cell: PFC) response *in vivo* to an exogenous antigen, sheep red blood cells. The mitogens concanavalin A (Con A), phytohemagglutinin (PHA), and lipopolysaccharide (LPS) were used to study *in vitro* lymphocyte activation, and a mixed-lymphocyte reaction (MLR) was used to test *in vitro* response to BALB/c alloantigens. Fluorescent-labeled antibodies, anti–Thy 1.2, $F(ab')_2$ goat anti–mouse IgG, anti-L3T4 (CD4) and anti–Lyt-2 (CD8), were used to identify lymphocyte phenotypes in a FACStar flow cytometer (Becton Dickinson).

Compared to normal controls, the number of PFC in spleens of homozygous β-thalassemic mice was reduced to 25% of normal, even though their spleens were 4 to 6 times larger than normal. Spleen cells from β-thalassemic mice also gave reduced responses to T-cell mitogens Con A and PHA (30–60% and 10–30% of normal, respectively) and in the MLR to an alloantigen (40–60% of normal) but a near-normal response (70–100% of normal) to a B-cell mitogen, LPS. FACStar analyses established that the percentages of Thy-1$^+$ T cells, IgG$^+$ B cells, and CD4$^+$ and CD8$^+$ T cells were reduced to approximately 40, 70, 65 and 35% of normal, respectively. The CD4/CD8 ratio was twofold higher in spleens of β-thalassemic mice compared to the level in controls. When cell fractions from nylon-wool columns

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were used to prepare cultures with similar numbers of Thy-1$^+$ lymphocytes, lymphocytes from β-thalassemic mice still gave a reduced mitogen response to Con A and PHA.

These studies established that β-thalassemic mice are immunodeficient. The PFC assay measures humoral response, but the induction of PFC depends on cellular interactions and the production of lymphokines. Excess iron might cause failure of macrophages to be effective antigen-presenting cells, which would disrupt the interleukin cascade that controls lymphocyte differentiation. Alternatively, aberrant iron metabolism might perturb lymphocyte subsets, which would interfere with immunoregulatory cellular interactions.[5]

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Somatomedin C and Zinc Status in β-Thalassemia Major

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A significant correlation between plasma somatomedin C (Sm-C) level and zinc status (plasma, bone, and liver Zn) has been reported in experimental studies.[1] Synthesis of Sm-C in the liver requires zinc.[1,2] Growth retardation is a prominent feature of zinc deficiency observed in man and animals.[1-4] Chronic zinc deficiency associated with hyperzincuria has previously been shown in β-thalassemia major in Turkey.[5] The present study was undertaken to investigate the effect of zinc deficiency on plasma Sm-C activity in this disorder.

Plasma Sm-C levels were measured by radioimmunoassay (RIA; Nichols Institute Products, Calif.), blood Zn levels (plasma, erythrocyte) and hair and bone Zn concentrations were studied by flame atomic absorption spectrophotometry in 12 thalassemia major patients (ages ranging from 1.5 to 14 years) and 20 controls.[4-6] Hematological studies were performed with standard techniques. Analysis of growth data revealed that more than half of the thalassemic children had growth retardation, as evaluated by height and by bone ages.

Plasma Sm-C concentrations were found to be decreased in 9 out of 12 thalassemic patients, with the levels below normal for age and sex (FIG. 1). At the same time,

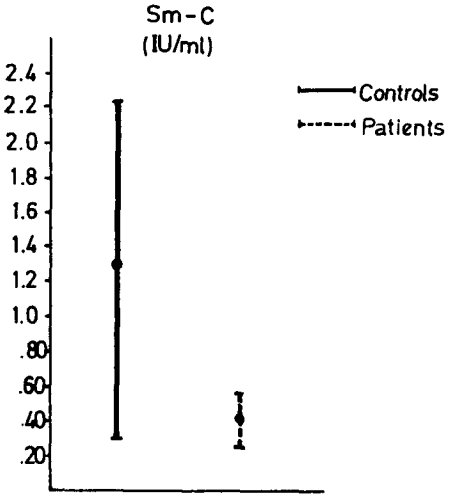

FIGURE 1. Mean plasma Sm-C concentrations in 20 controls (mean ± SD, 1.28 ± 0.96 IU/ml) and 12 β-thalassemic patients (0.40 ± 0.17 IU/ml). The difference between the two groups is significant ($p < 0.001$).

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TABLE 1. Somatomedin C and Zinc Status in Homozygous β-Thalassemia

	Mean ± SD				
Group	Plasma Sm-C (IU/ml)	Plasma Zn (μg/dl)	RBC Zn (μg/ml)	Hair Zn (μg/g)	Bone Zn (μg/g)
Patients (n = 12)[a]	0.40 ± 0.17[b]	77.8 ± 11.8[b]	12.6 ± 2.5[b]	114.2 ± 33.5[c]	110.8 ± 62.7[c]
Controls (n = 20)	1.28 ± 0.96	115 ± 23	18.4 ± 0.4	172.9 ± 58.5	40.3 ± 8.35

[a] n = 8 for hair Zn measurement and 10 for bone Zn.
[b] $p < 0.001$ compared to corresponding value for controls.
[c] $p < 0.01$ compared to corresponding value for controls.

blood (plasma and erythrocyte) and hair zinc levels were significantly decreased, reflecting chronic zinc deficiency (TABLE 1). Growth retardation is a common feature observed both in thalassemia and in zinc deficiency.[7] Furthermore, in our experience, zinc supplementation has promoted linear growth in β-thalassemia patients. This is probably the best indicator of zinc deficiency.[4,7] On the other hand, according to experimental evidence and recent human studies,[1-3] Sm-C synthesis in the liver depends on zinc. Although the mechanism for depression of plasma Sm-C levels in thalassemia remains obscure, zinc deficiency observed in our patients may play a role. In addition, defective hepatic biosynthesis of Sm-C due to iron overload may be an another contributory factor in thalassemia, since a significant correlation between iron overload and depressed Sm-C activity has previously been shown in this disorder.[8] The high concentration of zinc in bone was an unexpected finding in thalassemia patients. However, a similar feature has also been demonstrated in liver cirrhosis.[6]

In conclusion, the present study reveals that Sm-C decreases in β-thalassemia patients, who were also zinc deficient, suggesting a possible zinc dependence of Sm-C production in this disorder. Further study is required to analyze the effects of iron overload on Sm-C levels.

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Urinary Carboxyglutamic Acid and Serum Osteocalcin in Cooley's Anemia

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Carboxyglutamic acid (GLA) is an amino acid that binds calcium. It is derived from glutamic acid by reactions involving vitamin K and CO_2, in microsomes containing carboxylase. Osteocalcin (OC), bone GLA, is a protein containing three GLA residues; it binds calcium and is vitamin K dependent.[1] It is present in bone and in the blood.[1a] It is excreted by the kidneys as GLA, without degradation (FIG. 1). OC is a measure of bone turnover.[2,3] Its level in serum is elevated in Paget's disease of bone, in primary hyperparathyroidism, and in rickets; it is normal or slightly increased in osteoporosis. Urinary excretion of GLA is normal in Paget's disease, and increased in osteoporosis[4] (TABLE 1). Seven out of 10 patients with transfusion-dependent Cooley's anemia had elevated serum OC and urinary hydroxyproline, a measure of bone resorption (TABLE 2). The urinary GLA was, however, decreased, even in those given vitamin K (TABLE 3). These finding indicate high bone turnover and, an abnormality that has not been previously described in Cooley's anemia, a defect in the conversion of OC to GLA. This abnormality is in addition to an inability in Cooley's anemia to convert vitamin D to 25-hydroxy-vitamin D.[5,6] GLA binds to calcium with vitamin D, stimulating excretion of GLA. This inability to convert vitamin D is unlikely to cause decreased GLA excretion, since GLA was also decreased in one of our Cooley's anemia patients who had normal levels of 25-hydroxy-vitamin D and parathyroid hormone. There were patients with Cooley's anemia who had low serum alkaline phosphatase levels with elevated OC and decreased GLA. The serum calcium level was mostly in the normal range. About 20% of the urinary GLA is derived from OC and 80% from the vitamin K–dependent coagulation proteins;[1] almost all of the Cooley's anemia patients had normal coagulation tests. Some of the patients were given vitamin K without an increase in GLA urinary excretion. The decreased GLA excretion in Cooley's anemia may be due to the production of an abnormal undercarboxylated OC, low in GLA, by the

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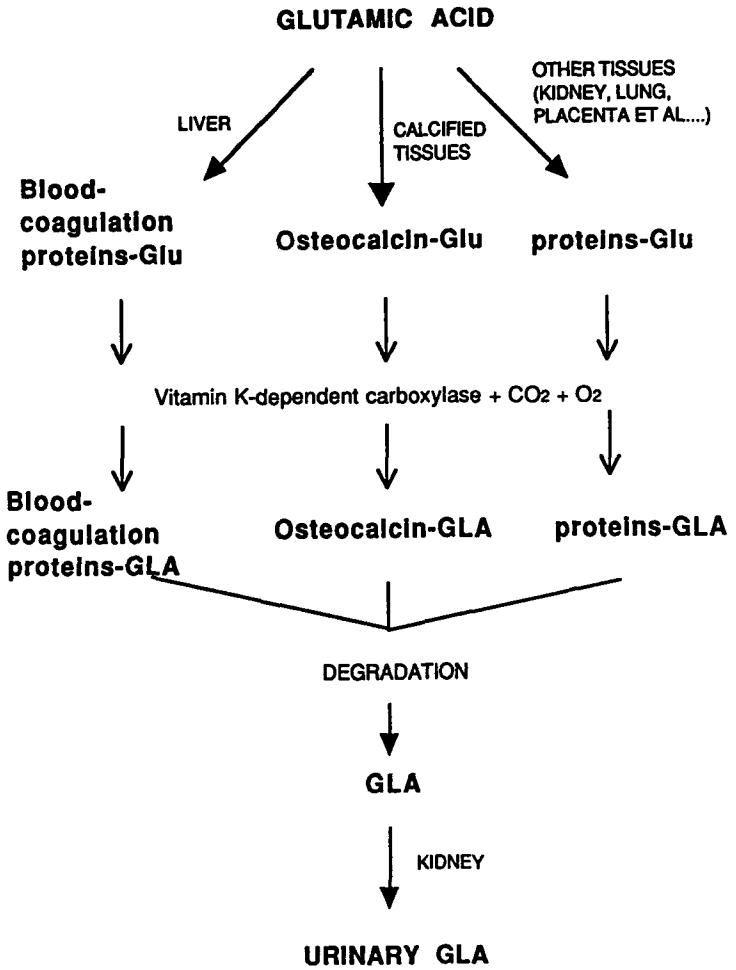

FIGURE 1. Pathways of carboxyglutamic acid (GLA) and osteocalcin formation, degradation, and excretion.

TABLE 1. Changes in Levels of Serum Osteocalcin (OC) and Urinary Hydroxyproline (OH-Pro) and Carboxyglutamic Acid (GLA) with Various Diseases

Disease	Serum OC	Urinary OH-Pro	Urinary GLA
Paget's disease	↑ ↑ ↑	↑	N[a]
Hyperparathyroidism	↑	↑	↑
Osteoporosis	↑ or N[a]	↑	↑
Cooley's anemia	↑	↑	↓

[a]N, normal.

TABLE 2. Urinary Carboxyglutamic Acid (GLA), Hydroxyproline (OH-Pro) and Creatinine and Serum Osteocalcin (OC) and Bone Alkaline Phosphatase (AP) in Patients with Cooley's Anemia

	Urinary			Serum	
Patient	GLA (μmol/24 h)	OH-Pro (mg/24 h)	Creatinine (mg/24 h)	OC (ng/ml)	AP (IU/l)
Cz	28.5	37.1	1030	11.7	22.8
Co	18.9	84.4	409	17.7	24.3
Ci	16.8	20.8	499	17.2	24.3
Gu	24.9	54.7	1040	10.4	8.7
Ma	15.1	115.3	772	29.6	21.5
Mi	30.5	28.5	1536	3.3	5.6
Or	21.1	49.5	540	16.8	16.9
Pa	21.1	92.4	1409	16.2	20.5
Normal range					
Age 15–20 yr	45 ± 15	40 ± 10	1000 ± 200	9 ± 3	15 ± 5
Adult	32 ± 8	25 ± 10	900 ± 150	4.5 ± 2	14 ± 5

TABLE 3. Effect of Vitamin K Supplementation on Urinary Carboxyglutamic Acid (GLA), Hydroxyproline (OH-Pro), and Creatinine Excretion in Patients with Cooley's Anemia

Patient[a]	GLA (μmol/24 h)	OH-Pro (mg/24 h)	Creatinine (mg/24 h)
Cz			
Before	26.2	54.5	1030
After	34.3	42.7	1200
Co			
Before	28.4	80.4	555
After	13.3	37.7	375
Mi			
Before	34.3	15.4	800
After	28.1	15.2	1500
Pa			
Before	21.1	92.4	1409
After	21.3	82.8	1594

[a]Before, after: before and after vitamin K supplementation.

osteoblasts. GLA in OC plays an important role in calcium transport, bone formation, and calcium homeostasis. If an abnormal OC is present, the protein would, depending on the GLA content, bind with difficulty or not at all with calcium. The postulated decrease in GLA synthesis may be contributing to the growth retardation[7] and secondary to the increased iron deposition present in Cooley's anemia.[8]

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Orthognatodontic Evaluation in Cooley's Disease

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In the past, the typical craniofacial alterations observed in patients with Cooley's disease were characterized by macrocephalia due to thickened diploe, prominent parietal and frontal cranial bones, a depressed nasal pyramid, prominent zygomatic bones, scanted palpebral rima, eyes turned downwards and to the outside, incisor proinclination, and maxillary prognathism.[1-3] In these patients, teleradiographic studies of the norma lateralis[1,4-7] showed a severe increase of the lower facial height, with alveolar anterior maxillary prominence, mandibular postrotation and skeletal class two (FIG. 1).

In teleradiographic studies of the norma lateralis carried out in 35 patients with Cooley's disease, aged between 3 and 33 years, we observed[8] a minimum severity of maxillofacial abnormalities and improved craniofacial growth in subjects who had undergone an intensive transfusional regimen for 4–5 years during the growth period.

Today, the early intensive transfusional regimen has almost completely prevented the characteristic maxillofacial alterations observed in subjects with thalassemia major (FIG. 2). However, the frequent presence of malocclusions in these patients led us to evaluate the possibility of orthodontic treatment.

Cephalometric studies were carried out in 50 subjects with thalassemia major, 24 males and 26 females, aged between 4.3 and 13.4 years. Since the first year of life, each had regularly undergone a transfusional regimen in order to maintain pretransfusion hemoglobin levels at 11 g/dl.

Facial types evaluated according to VERT gave results of meso in 54% of the patients, mild brachy in 8%, brachy in 12%, severe brachy in 2%, mild dolicho in 10%, dolicho in 12%, and severe dolicho in 2%. This distribution was similar to that observed in normal people by Ricketts,[9,10] indicating that modern intensive transfusional regimens prevent sagittal cranial alterations in Cooley's disease. Therefore, thalassemic patients with malocclusions can undergo orthodontic therapy similar to normal subjects.

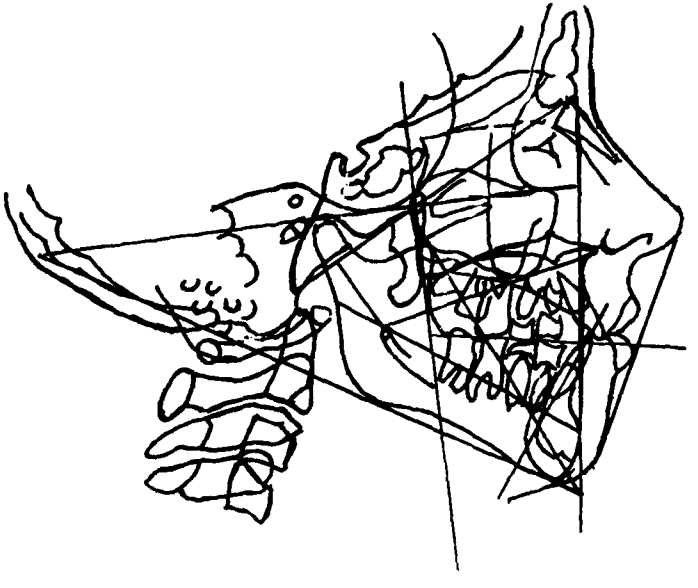

FIGURE 1. (*left*) Typical craniofacial alterations previously seen in patients with Cooley's disease, before the institution of an early intensive transfusional regimen.

FIGURE 2. (*above*) Maxillofacial alterations are now almost completely prevented by the early intensive transfusional regimen for patients with Cooley's disease. Compare to FIGURE 1.

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Protein S, Protein C, and Antithrombin III in Thrombotic Disease

Their Role in β-Thalassemia Major

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It is known that some patients affected by β-thalassemia major can develop central nervous system vascular lesions after blood transfusions. Recently several reports have demonstrated an activation of the coagulation system,[4,5] a reduction of both antithrombotic proteins (antithrombin III and protein S)[6] and an alteration of lipid status,[7] which can be considered predictive of a thrombotic risk. These alterations seem to be correlated with the liver damage always present in older thalassemic patients.[8,9] Protein S, another vitamin K–dependent protein, enhances the degradation of factors Va and VIIIa by activated protein C,[10] showing a modulator effect on coagulation. In order to establish the exact role of antithrombin III, protein C and protein S in the thrombotic risk of these patients, we studied these antithrombotic proteins in 77 polytransfused thalassemic patients.

Venous citrated blood samples were collected from 77 polytransfused thalassemic patients who were treated in our Cooley's Center of Catania. Plasma was frozen at −40°C and tested within a week. Twenty donors were choosen among healthy medical staff to represent a control group. Proteins C and S were measured by an immuno-Elisa method (Boehringer-Mannheim), and values in the range of 70–140% were considered normal. Antithrombin III activity was measured by a chromogenic method (Immuno), and values in the range of 80–120% were considered normal. Liver damage was assessed by means of serum transaminase levels (GPT, U/l). GPT values higher than three times the normal value (of 12 U/l) for more than 6 months were considered indicative of a chronic hepatitis. The study was approved by the regional ethical and scientific committee of the Cooley's Disease Association. Statistical analysis of the results was performed using a two-tailed Student's t test and Mann-Whitney test. p values less than 0.05 were considered to be statistically significant. Regression lines were calculated by the method of least squares, and the correlation coefficient (r) was calculated for each group.

In all patients we found a marked reduction of protein C (mean value, 35.73; range, 14–66) and a mild reduction of protein S (mean value, 54.17; range, 111–25). The antithrombin III, expressed as a percentage of normal activity, was also reduced in the majority of patients (mean value, 61.83; range, 18–100). The distribution of normal and abnormal values observed was low values of protein C in 100% of patients, low values of protein S in 66.7% and low values of antithrombin III activity in 55.4%. We found a significant difference for proteins C and S between splenectomized and non-splenectomized patients ($p < 0.05$; TABLE 1). Moreover, both proteins C and S were found lower in patients with higher levels of serum transaminases (>3 times the normal value; TABLE 1). An inverse correlation was found between

TABLE 1. Correlation between Splenectomy or Serum Transaminase (GPT) Values and Protein S and Protein C Levels

Variable	Correlation	
	Protein S (%)	Protein C (%)
Splenectomy		
Splenectomized (n = 27)	62.98 ± 23.95	21.77 ± 17.64
Non-splenectomized (n = 50)	52.54 ± 17.87[a]	32.60 ± 22.70[a]
Serum Transaminases		
GPT < 36 U/l (n = 40)	59.65 ± 22.23	40.54 ± 24.45
GPT > 36 U/l (n = 37)	52.54 ± 17.87[b]	24.15 ± 18.64[b]

[a]Difference from splenectomized patients significant (Student's t test), $p < 0.05$.
[b]Difference from patients with GPT values < 36 U/l (Student's t test), $p < 0.05$.

proteins C and S (FIG. 1). In contrast, we did not find any difference with regard to chronic hepatitis and splenectomy for antithrombin III levels.

The higher survival of thalassemic patients by the use of a high transfusional regimen has increased the risk of cirrhotic evolution due to chronic infectious hepatitis and post-transfusional siderosis.[8,9] Moreover, a rapid increase of hemoglobin with transfusion; the alteration of coagulative vitamin K factors, antithrombotic,

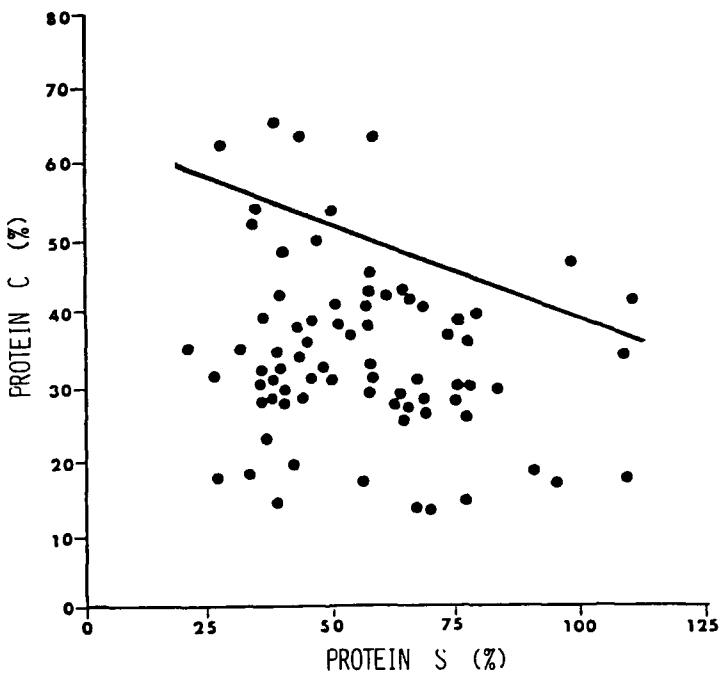

FIGURE 1. Inverse correlation between protein C and protein S in polytransfused thalassemic patients.

fibrinogen and fibrinolytic systems;[12,13] and the alteration of lipid status[7] represent a real risk for intravascular coagulation in older patients. In this regard it is known that antithrombin III plays a main role as an inhibitor of activated coagulation factors, and reduced antithrombin III activity correlates with chronic intravascular coagulation in cirrhotic liver,[11] increasing the fibrinogen turnover. In this study the activities of antithrombin III and protein C were found to be reduced, showing a thrombophilic status which may be precipitated by causes such as surgery, immobilization, rapid correction of hematocrit, etc. However, the percentage of patients affected by vascular accidents, despite low levels of antithrombotic proteins,[6] is low. Protein S, which was found to be present at quite normal levels may represent in these patients an important factor controling thrombotic risk. Francis *et al.*[15] reported reduced levels of both proteins C and S in sickle cell anemia, which correlated with increased thrombin activity and increased fibrin formation. In our study the reduction of protein C and protein S correlated with liver damage as assessed by transaminase values, since both protein C and protein S are produced by the liver. Moreover, the inverse correlation between proteins C and S may be explained by reduced consumption of protein S, a cofactor of protein C, in the presence of low levels of protein C. In conclusion, this study underlines the role of antithrombotic proteins on the thrombotic risk in thalassemic patients, where thrombin activation modulates both antithrombin III activity and proteins C and S catabolism. In the presence of a low level of protein C, normal levels of protein S and antithrombin III represent the main protection mechanism to inhibit the procoagulant factors and protect these patients. However, this situation may be associated with higher thrombotic risk in older patients with lower values of antithrombin III activity and protein S. We suggest a specific monitoring of older patients, especially if their liver biopsy shows a cirrhotic status.

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Interferon-α Therapy in Thalassemic Patients with Chronic Non-A, Non-B Hepatitis

Preliminary Results and Proposed Protocol

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More than 90% of post-transfusional chronic hepatitis is due to hepatitis C virus,[1,2] and the estimated incidence of infection among persons receiving transfusions is 5–10%.[3] Chronic hepatitis develops in at least half of the patients with acute non-A, non-B hepatitis, and cirrhosis develops in at least 20% of them.[4] There is currently no therapy for chronic hepatitis, but several reports have suggested that interferon-α may be useful in the treatment of patients with chronic non-A and non-B hepatitis.[5,6] On the basis of these data, a randomized, controlled trial has been conducted to assess the efficacy of recombinant interferon-α 2a in thalassemic patients with chronic hepatitis C.

Four thalassemic patients, aged 7–15 years, were randomly assigned to treatment with recombinant interferon-α2a (Roferon A, Roche), administered by the intramuscular route three times a week at a dosage of 5×10^6 U/m^2 for 6 months and at a dosage of 3×10^6 U/m^2 for another 6 months. Four other thalassemic patients were assigned to receive no treatment. The criterion for a complete response was defined

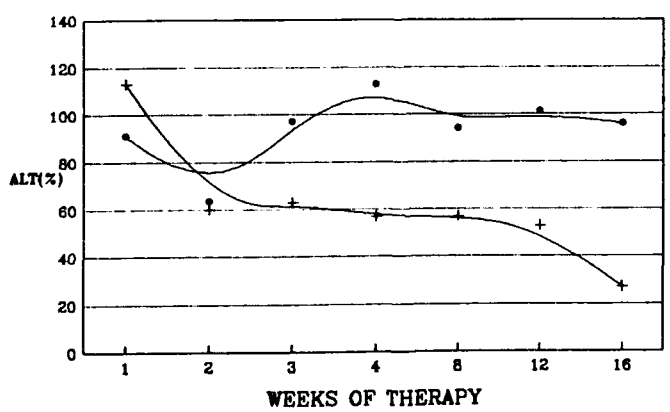

FIGURE 1. Effect of treatment with interferon-α on serum alanine aminotransferase (ALT) levels in chronic hepatitis. The % change in serum ALT for interferon-treated patients (+; 5×10^6 U/m^2, 3 times per week) and for controls (●) is shown.

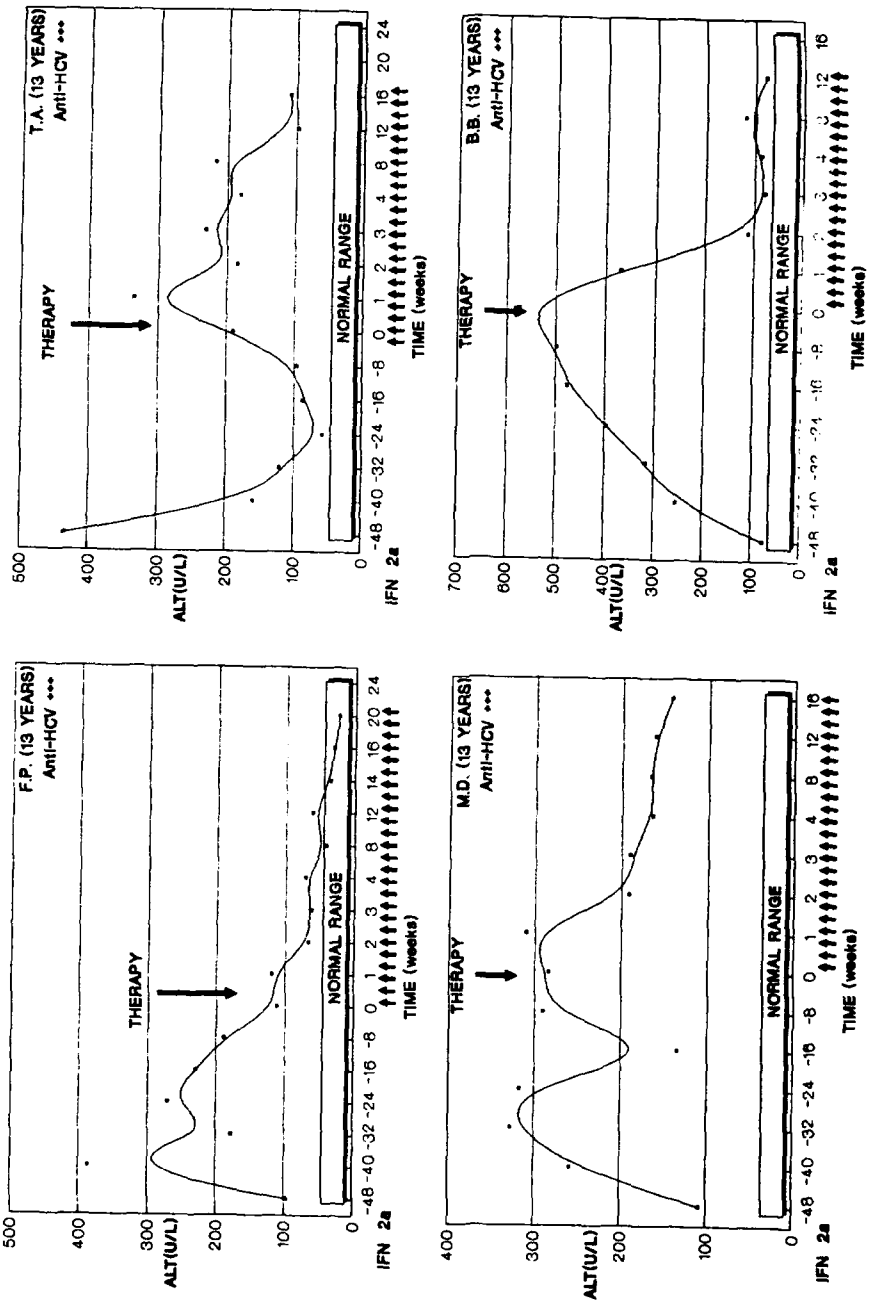

FIGURE 2. Serum ALT values in interferon-treated chronic hepatitis patients. Period of treatment for each patient, at 5×10^6 U/m^2, 3 times per week, is indicated by *arrows* (↑).

as the normalization of the serum alanine aminotransferase (ALT) level. In all treated and untreated patients, a liver biopsy was performed before randomization. The biopsy specimens were graded with respect to degree of periportal, portal and lobular inflammation and fibrosis according to the Knodell scoring system. A second liver biopsy will be performed at the end of treatment. The presence of antibody to the hepatitis C virus was determined in the serum samples before the treatment by a solid-phase antibody capture radioimmunoassay (ORTHO).[7]

The pretreatment biopsy showed-hepatitis C virus chronic active hepatitis in all patients, who were anti-hepatitis C virus positive. A decrease of ALT was observed in all treated patients, while in the untreated we did not find any improvement (FIG. 1). The serum ALT declined to normal (a complete response) in one of the four treated patients within two months from the beginning of therapy (FIG. 2). Headache and fever occurred only in the treated patients, but these symptoms disappeared after the first doses of interferon. No patients had to interrupt the treatment because of the appearance of side effects.

Our preliminary results in a short period of time (4 months) indicate that interferon therapy is useful in thalassemic patients with chronic hepatitis C because, it certainly reduces liver cells necrosis as monitored by ALT values. Moreover, this study seems to demonstrate that interferon-α at a dosage of 5×10^6 U/m^2 in thalassemic children is well tolerated. However, it seems that half of the patients who responded to interferon therapy will have a relapse within six months after the end of treatment.[8] Because of this experience, our protocol of therapy established a period of treatment of 12 months with two different dosages: 5×10^6 U/m^2 for the first 6 months and 3×10^6 U/m^2 for the second 6 months. The effects of interferon therapy on the long-term natural history of chronic hepatitis C in thalassemia remain to be defined, and only a follow-up after the end of treatment can confirm our preliminary encouraging results in these four thalassemic patients.

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The New Synthetic Hepatitis B Vaccine Administered at Low Doses by the Intradermal Route in Subjects at High Risk of Hepatitis B

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Only the use of a program of mass vaccination against hepatitis B, especially in highly endemic areas for hepatitis B, could stop this severe infectious disease. However, the high number of subjects that should be vaccinated considerably increases the cost of this program. In reference to this problem in other trials, it was found that the hepatitis B virus (HBV) vaccine (HB-VAX, Merck Sharp and Dohme) administered at low doses (2 μg) by the intradermal route was immunogenic.[1-4] A less expensive and safer vaccine, prepared by a recombinant DNA technique (Engerix B, Smith Kline Biological) has recently been introduced in Italy. Since there is no experience with its administration by the intradermal route at low doses in subjects at risk, we have investigated this possibility in different classes of subjects at risk (patients with thalassemia major, sickle cell anemia or neurological handicaps, and healthy medical staff).

In this study, 39 subjects—healthy medical staff, 9 patients with neurological handicaps, 5 patients with thalassemia major, and 5 patients with sickle cell anemia—were enrolled. The vaccine, Engerix B (Smith Kline Biologicals), was administered at doses of 5 μg in 0.20 ml intradermally on the volar surface of the forearm. All patients were observed 48 h after vaccine inoculation to assess the delayed tissue hypersensitivity (DTH) reaction. The DTH reaction was considered to be positive if a visible cutaneous macula larger than 1 cm^2 was found. Two weeks after every vaccine dose, we collected serum samples to assay the anti-HBs titer; and we continued the vaccine inoculations at intervals of two weeks up to the fourth dose. HBs antibody levels were measured by radioimmunoassay (Ausab-Abbott Laboratories), with a titer > 19 U/l considered to be positive.

All our subjects showed a DTH reaction 48 h after the second dose of vaccine. The small pigmented macule became larger when we gave the third and fourth vaccine doses. Moreover, every time we gave the intradermal dose we observed a reappearance of a macula in the sites where we had applied the previous vaccine doses.

In all our subjects the DTH reaction became positive (>3 mm) before the anti-HBs levels were higher than 10 U/l. All our subjects (100%) showed a positive anti-HBs titer after the fourth dose of the vaccine (45 days after the first dose of vaccine) (FIG. 1). The anti-HBs titer was higher than 1,000 U/l in many subjects

(84.4%; TABLE 1). No difference was found in the final titer of anti-HBs (> 1,000 U/l) among the four groups of patients.

The antibody response to vaccine administered by the intradermal route at low doses (2 μg) was found comparable to that obtained by the standard intramuscular route (20 μg) in 32 patients with thalassemia and sickle cell disease, as reported by Mok *et al.*[4] Recently in a study with hepatitis B vaccine (HB-VAX Merck-Sharp and

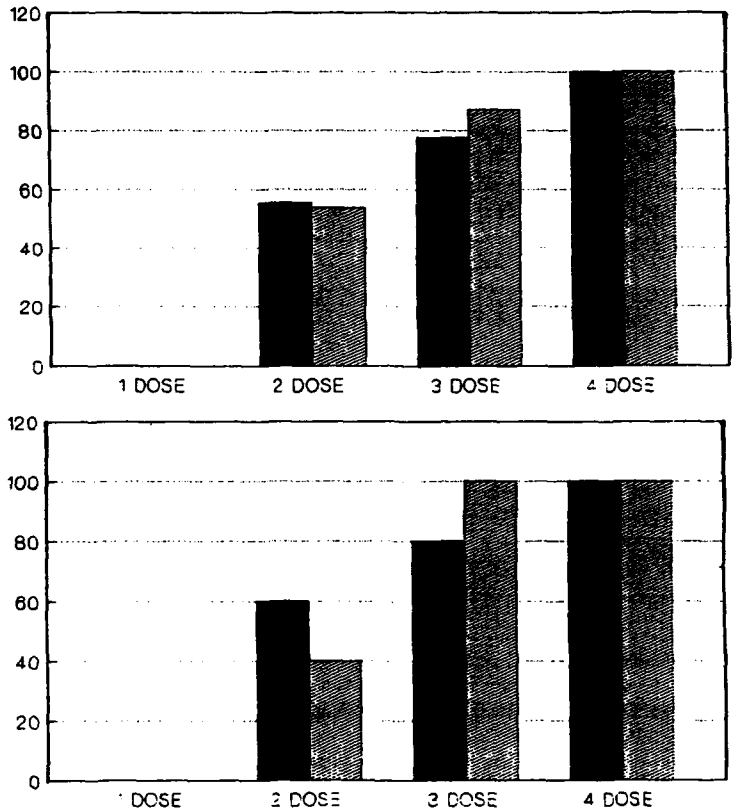

FIGURE 1. Percentage of subjects with anti-HBs titer > 10 U/l after intradermal vaccination with the recombinant DNA vaccine. Titers were determined 14 days after administration of each dose of vaccine. **(Upper panel)** Response of patients with neurological handicaps (*solid bars*) and of healthy medical staff (*stippled bars*). **(Lower panel)** Response of patients with thalassemia major (*solid bars*) and of those with sickle cell anemia (*stippled bars*).

Domhe) we have reported[5] on a vaccination program by the intradermal route at low doses which has the advantages that three doses of vaccine are saved and that all unresponsive patients can be revaccinated to get a positive immune response. The positive results reported here with the new genetically engineered hepatitis B vaccine confirm again the effectiveness of the intradermal route for vaccination against HBV and indicate that this new vaccine can also be administered by the

TABLE 1. Range of Anti-HBs Antibody Titers after Intradermal Vaccination

Anti-HBs Titer (U/l)	Number of Subjects			
	1 Dose	2 Doses	3 Doses	4 Doses
<10	—	23	9	—
>10, <100	—	7	3	—
>100, <512	—	19	23	5
>512, <1000	—	1	5	5
>1000	—	8	18	49[a]

[a]84.4% of subjects gave this titer after 4 doses of the vaccine.

intradermal route at low doses. It is therefore suitable to use in a mass vaccination program. Although some authors[6,7] described lower antibody levels after vaccination by the intradermal route, we observed an increase of the anti-HBs titer above 1,000 U/l in many subjects (84.4%) of the four different groups, using the new genetically engineered vaccine. So more than 90% of them should have antibody levels higher than 10 U/l after 5 years.[8,9] Finally, there are two remaining major questions relating to the use of the new hepatitis B vaccine in this way: how long will the protection last and when should booster doses of the vaccine be given? Only vaccination and follow-up of a large group of subjects will confirm the efficacy of this cheap vaccination protocol against HBV.

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Changes in Spin-Lattice (T1) and Spin-Spin (T2) Relaxation Times of Packed Red Blood Cell Samples during Sixty Days at Room Temperature

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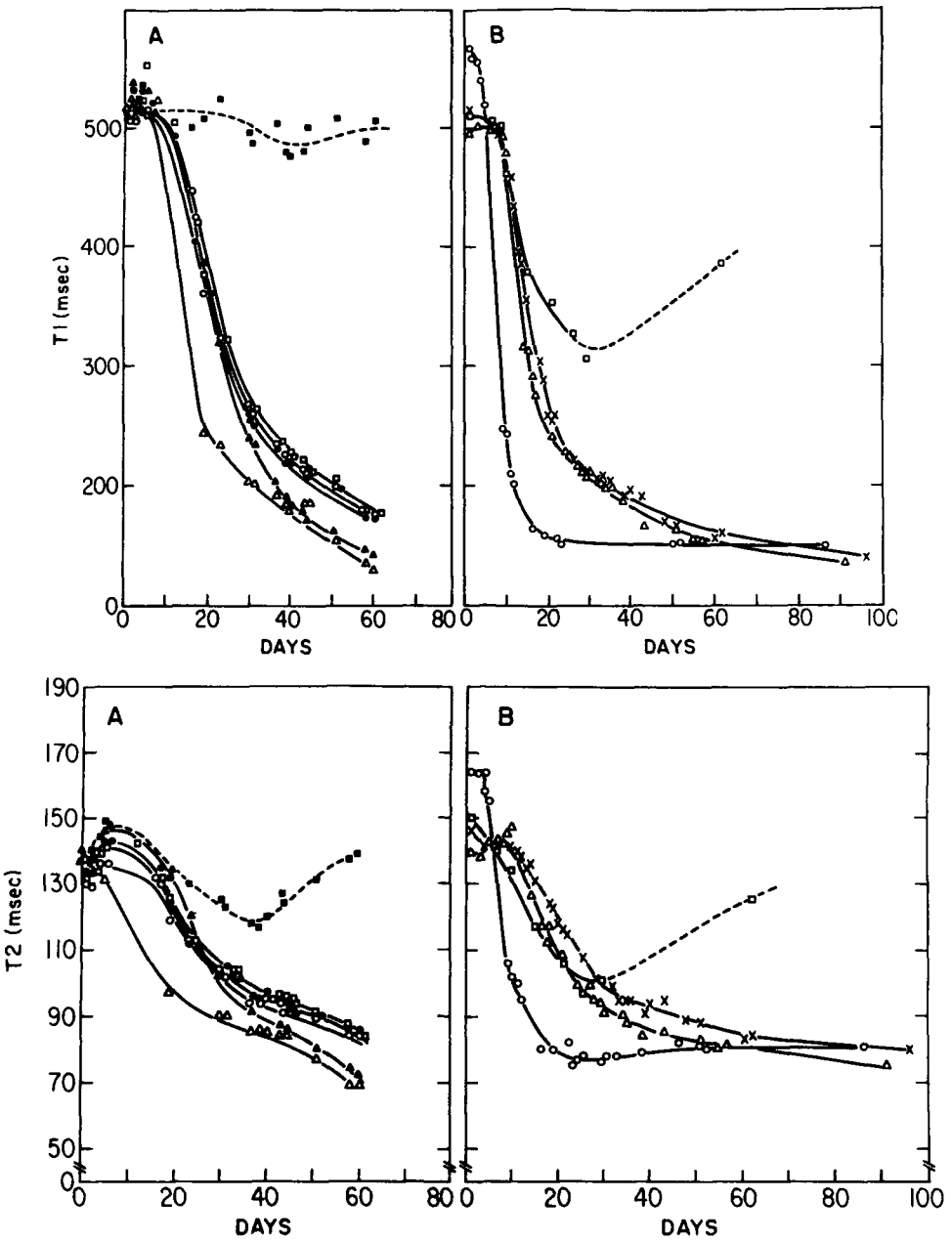
In a functioning red blood cell (RBC), the heme-iron moieties of its deoxyhemoglobin exist in the Fe^{2+} oxidation state. The iron atoms of a methemoglobin molecule, however, correspond to their respective Fe^{3+} oxidation states. Such a ferri-heme moiety cannot bind to an O_2 molecule. When the entire hemoglobin content of an RBC gets converted to methemoglobin, the cell becomes non-functional until some or all of its methemoglobin is converted back into deoxyhemoglobin.

For each assay, venous blood from an adult human was mixed with sodium heparin immediately after withdrawal and centrifuged for 6 min in a Fisher Centrific Model 228 centrifuge within 2 h of collection. The separated plasma layer, along with the buffy coat, which was at the top of the hematocrit layer, was removed and discarded. The remaining cellular material in the tube corresponded to packed red blood cells (PRBC). While its further exposure to air was limited to a minimum, the PRBC content was carefully mixed with a Pasteur pipet. A 0.5-ml sample of the homogeneous mixture of PRBC was transferred to an NMR sample tube (103 × 10.25 mm). A rubber stopper on top of the tube both enclosed and isolated the sample. The spin-lattice (T1) and spin-spin (T2) relaxation times of its water protons were determined in a Seimco Micro-pulse NMR spectrometer (New Kensington, Pa.) at 13.2 MHz and 29°C. The T1 of deionized water in this defined system approximated 3,000 msec, and its T2 was about 2,000 msec.

Six PRBC samples were processed from a single blood specimen of a male donor. T1 and T2 changes in each during 60 days at room temperature are indicated in FIGURES 1A and 2A, respectively. While the T1 of one such sample shortened from 517 msec to 142 msec, its T2 shortened from 136 msec to 72 msec. That corresponded to a decrease in its T1/T2 ratio from 3.8 to ca. 2 during the 60-day period.

Four PRBC samples—two from males and two from females—comprised the comparison group. The T1 and T2 changes in each of these during the experimental period are indicated, respectively in FIGURES 1B and 2B. While T1 of one of them shortened from 515 msec to 156 msec, its T2 shortened from 146 msec to 83 msec. These shortenings of T1 and T2 decreased its T1/T2 ratio from 3.5 to ca. 2.

For each one of the two PRBC samples described above, the resulting T1/T2 ratio of ca. 2 indicated conversion of the hemoglobin content into methemoglobin during the period of investigation.



FIGURES 1 and 2. Changes in T1 (**FIG. 1:** ***upper panels***) and T2 (**FIG. 2:** ***lower panels***) during the experimental period: (**A**) 60 days of monitoring the study group comprising sample 1 (○), sample 2 (●), sample 3 (□), sample 4 (■), sample 5 (△), and sample 6 (▲); (**B**) 90 days of monitoring the comparison group consisting of sample 7 (○), sample 8 (□), sample 9 (△), and sample 10 (×). Microbial contamination provided to the NADH cytochrome b_5 reductase the required energy to function in the reduction of methemoglobin to deoxyhemoglobin in sample 4; the manifestation of this process from T1 measurements was also apparent a few days later in sample 8. In comparable samples, the time-course seen for sample 8 was more often observed.

Preliminary Efforts to Modify Iron Deposition in Homozygous β-Thalassemic Mice[a]

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Murine β-thalassemia was first detected by Lewis *et al.*[1] during screening for mutagenesis. We have previously compared normal (+/+), heterozygous (+/*th*), and homozyogous (*th*/*th*) mice to one another and to their human counterparts.[2] We find that homozygous β-thalassemic mice have a hypochromic, microcytic anemia comparable to thalassemia intermedia. Normal and heterozygous mice are essentially indistinguishable. Homozygotes are *not* transfusion dependent, despite the anemia; iron accumulates in their organs in the *absence of transfusions.* To learn whether additional parallels to iron loading in Cooley's anemia exist, we have attempted to modify iron deposition by maneuvers relevant to the situation in patients. Transfusions of normal and "effete" (heat-treated) blood potentially increase iron levels, while chelation with desferrioxamine (DFO) and desferriferrithiocin (DFFT) potentially decreases them. All maneuvers involved small numbers of mice and "low-intensity" studies, so all results must be regarded as preliminary.

Homozygous (*th*/*th*) mice received 0.5 ml of blood intraperitoneally under ether anesthesia weekly for 4–8 weeks. To distinguish the effects of transfusions which served both as a source of iron and a means of slowing endogenous erythropoiesis from those of transfusions which served only as a source of iron, some mice received normal blood and others received "effete" blood (FIG. 1), which was produced from normal blood by heating it to 56 °C for 110 sec. Blood smears, reticulocyte counts, blood counts, and hematocrits were monitored to establish the effectiveness of normal transfusions and the ineffectiveness of effete blood at inhibiting erythropoiesis. Non-heme tissue iron was extracted and quantitated colorimetrically.[3] Tissue sections were also prepared, stained with Gomori's Prussian blue, and semiquantitatively evaluated on a Leitz TAS Image Analyzer or counterstained and scored subjectively by two "blind" observers. Results for both methods of assessing the tissue sections agreed closely with the tissue iron quantitations; only the quantitations are shown. Splenic and hepatic iron stores were increased ($p = 0.0005$ and $p = 0.04$, respectively, by ANOVA) by eight transfusions with normal blood, while kidney and heart levels were unaffected (FIG. 1). All four organs had elevated iron stores after 4–6 transfusions with effete blood ($p = 0.0003$, $p = 0.005$, $p < 0.0001$, and $p = 0.0011$ for spleen, liver, kidney, and heart, respectively, by ANOVA). The transfusions with normal blood led to a marked decrease in splenomegaly, so that total body iron stores were only modestly affected. Transfusions with effete blood did *not* markedly diminish splenomegaly, so that total body iron stores were increased.

[a]This work was supported by a grant from the Cooley's Anemia Foundation.

Iron stores of Cooley's anemia patients are currently removed by subcutaneous injection of DFO (Desferal) via pump or, in noncompliant patients, by intravenous infusion. Given the obvious promise of the oral chelator DFFT,[4] we also chose to examine its effects. Both DFO and DFFT were kindly supplied by CIBA-Geigy.

We tested the β-thalassemic mouse model for its response to intramuscular injections of 2 mg/kg of DFO semiweekly. (If we had been aware of the existence of Alzet pumps, we would have used continuous subcutaneous infusion as the route.) The decrease in renal iron with DFO (FIG. 2) was significant ($p = 0.026$ by ANOVA), so one is encouraged to suspect that better methods of administration and higher doses of DFO would mimic the effectiveness seen for DFO in Cooley's patients. In the absence of continuous infusion and at such a low dosage, it is not surprising that splenic, hepatic and cardiac levels of iron were unaffected by DFO.

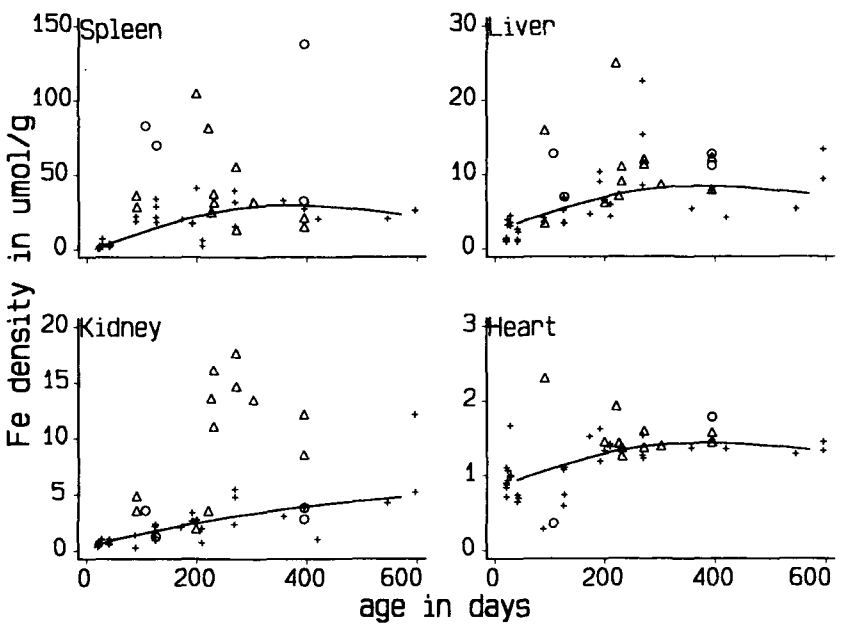

FIGURE 1. The effect of transfusions with normal (○) or effete (△) blood on tissue iron for homozygous β-thalassemic mice. Iron deposition increases with age in *th/th* mice[2]; hence, tissue iron densities are shown as a function of age. Data for the untransfused controls (+) have been fitted by a cubic spline procedure.

The studies with DFFT were hampered by unexpected chronic toxicity. Chronic oral DFFT proved lethal, despite a switch from gavage administration at 50 mg/kg twice weekly to *ad lib* administration in the water. To rule out misadventures during repeated ether anesthesia and gavage, we tried administering DFFT at 0.2 mg/ml in iron-free drinking water; based on *ad lib* consumption, this level yielded a dose of 200 mg/kg/week. Another series was started on 0.2 mg/ml DFFT in drinking water; but this also involved deaths, so the dose was decreased to 50 μg/ml. Surviving mice (FIG. 2) exhibited decreased tissue deposition of iron in the kidney ($p = 0.05$ by ANOVA).

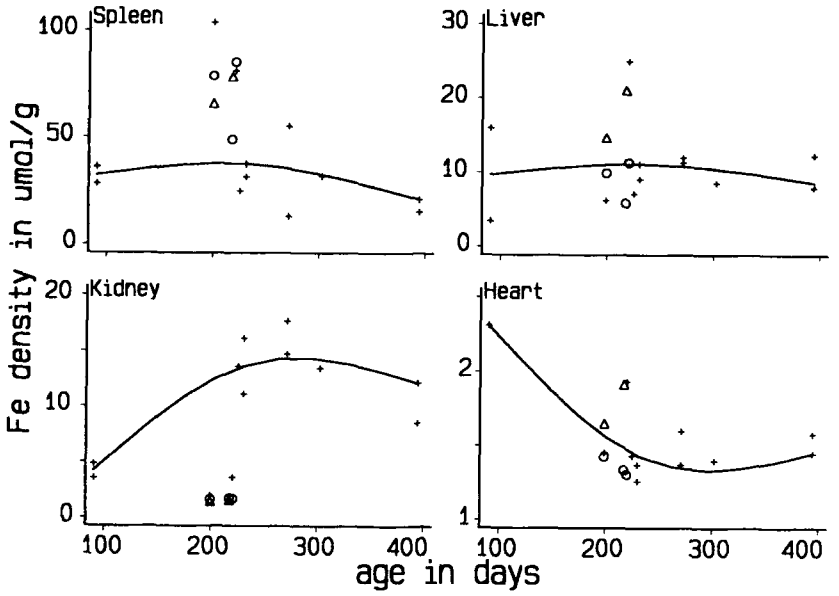

FIGURE 2. The effect of chelation by DFO (○) or DFFT (△) on tissue iron for effetely transfused homozygous β-thalassemic mice. Tissue iron densities are shown as a function of age, and the data for effetely transfused controls (+) have been fitted by a cubic spline procedure similar to that used in FIGURE 1.

All chelated mice appeared to be thriving until they either lost or failed to gain weight for the last week of life.

From these studies we make the following conclusions:

1. Homozygous β-thalassemic mice respond to transfusions with normal blood by exhibiting increased splenic and hepatic iron in the absence of a large increase in body iron stores.
2. Homozygous β-thalassemic mice respond to transfusions with effete blood by exhibiting increased tissue iron densities and increased body iron stores.
3. Homozygous β-thalassemic mice respond to DFO in a fashion that may resemble that in human patients.
4. The new oral chelator DFFT is toxic when administered chronicly in homozygous β-thalassemic mice. *Extreme caution should be applied to its use in humans* although the toxicity could be less in iron-overloaded mice than in normal mice.
5. The β-thalassemic mouse is valuable for comparisons of iron metabolism to that in patients with Cooley's anemia.

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The Belgrade Rat as a Model of Hepatic Transfusional Iron Overload in Cooley's Anemia

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Hepatic transfusional iron overload (TIO) is one of the complications that follows treatment of Cooley's anemia. The Belgrade rat has potential as a model for Cooley's anemia because it has a hypochromic, microcytic anemia inherited as an autosomal recessive trait (*b/b*). However, the liver of this animal has not been well characterized in terms of hepatic iron homeostasis. It is therefore the aim of this project to examine iron deposition in the liver of the Belgrade rat, with a view to using this animal as a model of TIO. In this study, animals have been iron loaded, using iron dextran, and the variations in hepatic iron deposition among homozygote and heterozygote rats examined by morphologic techniques.

Siblings from the cross of male homozygous (*b/b*) and female heterozygous (*+/b*) Belgrade rats, body weights 110–250 g, were randomized into three treatment groups. One group was treated intraperitoneally (i.p.) with 500 mg/kg body weight iron dextran (Imferon); the second group received this, followed by 100 mg/kg desferrioxamine (Desferal); and the third group received desferrioxamine alone. Male Sprague-Dawley rats (175–200 g) were also studied, following similar iron loading. Seven days after the last injection, the animals were sacrificed, and the livers excised and fixed by immersion in neutral-buffered formalin (Sigma). Livers were embedded in paraffin, sectioned at 4 μm, dewaxed, and stained using the Gomori modification of Perl's acid ferrocyanide method for non-heme iron, with a neutral red counterstain. The sections were examined microscopically and scored for location (periportal or pericentral), type (diffuse or granular), and quantity of iron in the various cells and tissues. Aliquots of the blood, taken at the time of sacrifice, were allowed to clot at room temperature; and the serum was aspirated for determination of serum transferrin by single radial immunodiffusion and for other serum iron parameters using a commercial kit (Sigma).

All livers from iron-treated animals showed heavy deposits in the Kupffer cells, with lesser deposits in the hepatocytes (principally pericentral). In males, iron deposition was lowest in the homozygous Belgrade rats (*b/b*) and highest in the livers of Sprague-Dawley rats. Livers from female Belgrade rat homozygotes, however, contained as much iron as the Sprague-Dawley rats; and livers from the female heterozygotes contained the heaviest iron deposits. The hepatic iron stores could be

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TABLE 1. Iron-loaded Livers

Rat	% Mural Cells Containing Fe		Proportionate Size of Kupffer Cells with Fe		% Hepatocytes Containing Fe		Fe in Hepatocytes[b]		Fe in CT Cells of Portal Tract[a,b]
	CV[a]	PA[a]	CV[a]	PA[a]	CV[a]	PA[a]	CV[a]	PA[a]	
Belgrade									
Male (+/b)	68	70	0.5	0.5	90	70	2.8	1.8	+1.0
Male (b/b)	26	45	0.2	0.2	62	81	2.3	2.7	−0.3
Female (+/b)	88	90	0.6	0.7	90	90	3.3	3.0	+1.0
Female (b/b)	34	69	0.2	0.3	90	90	2.0	2.0	−0.5
Male (+/b) + DFO[c]	54	59	0.4	0.5	17	13	1.0	0.5	+1.0
Male (b/b) + DFO[c]	0.2	2	0.1	0.2	0	6	0	1.5	−0.5
Male Sprague-Dawley	65	82	0.4	0.5	87	79	2.2	1.3	+0.9

[a]CV, pericentral; PA, periportal; CT, connective.
[b]Scored on a scale of −1 to 4.
[c]DFO, desferrioxamine treatment.

more readily mobilized by desferrioxamine from homozygous male livers than from those of the corresponding heterozygotes. Iron in the hepatocytes tended to be deposited more heavily in pericentral as opposed to periportal cells in male *+/b* and male Sprague-Dawley rats. This differential deposition was less marked in female *+/b* and absent in male and female *b/b* rats. Deposition of iron in Kupffer cells is greater in the area of the portal tract than in the area of the central vein (TABLE 1). The amount of iron in the hepatocytes and Kupffer cells decreases with desferrioxamine treatment and the percentage of cells containing iron also decreases with this treatment, as may be seen in TABLE 1. Serum iron parameters, however, did not appear to correlate with hepatic iron content, as may be seen in TABLE 2. There was a tendency for male *b/b* rats to have a low serum iron saturation. All except the male *+/b* animals had depressed serum iron values, in spite of Imferon treatment.

From these data it can be seen that the Belgrade rat has potential as a model of hepatic transfusional iron overload. The different patterns of iron deposition seen

TABLE 2. Serum Iron Parameters

Rat	Transferrin (g/l)	Serum Fe (mg/l)	% Saturation
Belgrade			
Male (+/b) + Fe	2.80	3.28	51
Male (b/b) + Fe	2.70	1.27	34
Female (+/b) + Fe	4.00	1.30	40
Female (b/b) + Fe	6.80	1.33	45
Male (+/b) + Fe/DFO[a]	2.60	1.19	57
Male (b/b) + Fe/DFO[a]	5.30	1.19	33
Male (+/b) + DFO	3.00	1.59	32
Male (b/b) + DFO	10.00	1.24	30
Male Sprague-Dawley	2.80	2.96	50

[a]Desferrioxamine (DFO) treatment given 7 days after last Imferon injection.

among the male and female *b/b* and *+/b* rats, together with the preliminary results showing differences between *b/b* and *+/b* animals in the chelation studies, suggest that these animals can be used to mimic the various forms of iron loading that are seen clinically in the Cooley's anemia patient and to clarify the possible modulating effects of sex and genetic factors on parenchymal iron deposition. Further studies are also required to elucidate the effects of iron loading on iron homeostasis in the Belgrade rat.

Non-Invasive Evaluation of Iron Load and Clearance in Patients with β-Thalassemia[a]

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Long-term chelation treatment with slow, subcutaneous infusion of desferrioxamine (Desferal, DFO) is the major treatment to prevent and reduce heavy iron load and to prolong the life-span of multitransfused thalassemic patients.[1-3] Since routine invasive sampling for iron in biopsies from internal tissues is not practical for a follow-up of chelation treatment, non-invasive approaches for the evaluation of parenchymal iron load are needed.

Computerized single- or dual-energy X-ray tomography,[4] nuclear resonance scattering,[5] and magnetic susceptibility[6] were among the non-invasive methods which were suggested. These methods suffer from limited sensitivity and difficulties in the differentiation between parenchymal and reticuloendothelial iron. Liver seems to contain high iron stores and may buffer their peripheral distribution,[1,7] which could trigger the complications found in the function of other internal parenchymal organs. But at the early stage the extremely high iron level in the liver will not reflect the possible siderosis in other tissues.

Diagnostic X-ray spectrometry (DXS) is a technique which was developed for the non-invasive analysis of major trace elements such as iron, zinc, and copper in external tissues with a sensitivity down to parts per million (ppm, μg/g wet tissue).[8-12] The method was previously described in detail.[8,11] It is based on the excitation of an area of external tissue a few square millimeters in size by low intensity monochromatic soft X-rays of 11.4 KeV and the detection of the specific X-ray fluorescence of the various elements by a liquid nitrogen–cooled solid state detector of high sensitivity and large active area (SR. 200, Jordan Valley Applied Radiation Industries, Migdal Haemek, Israel). The spectrum of the elements is processed, and the ratio between the X-ray fluorescence of each element and the scatter of the incident excitation beam allows accurate calculation of the concentration of each element (FIG. 1).

We have previously suggested that the skin could serve as a direct indicator of the non-specific deposition of iron in parenchymal tissues in β-thalassemics and other iron-loaded patients.[11] By examination of the palm thenar eminence, where the epidermis is thick, the epidermal Zn and Fe index could be evaluated. In the lateral flexor area of the arm, where the epidermis is thin, the readings represent the dermal

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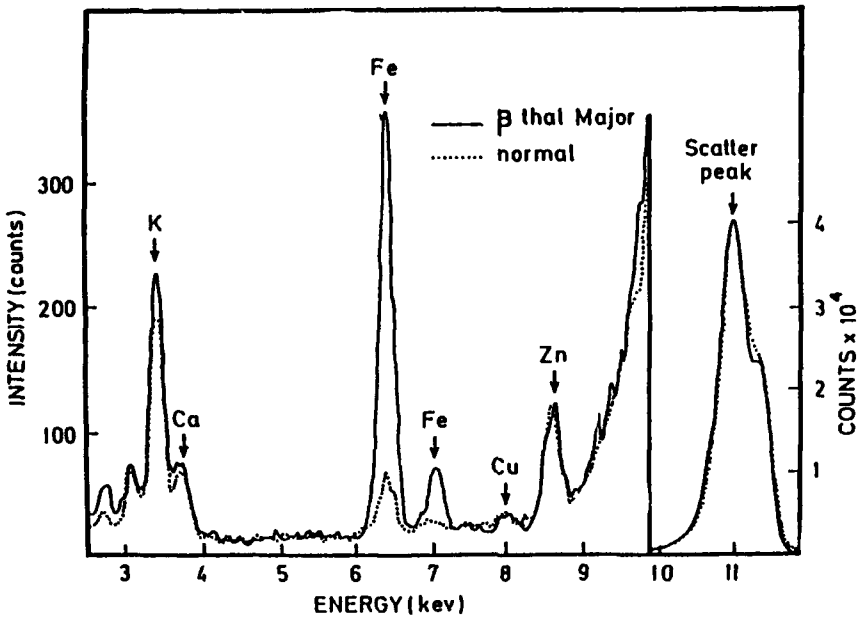

FIGURE 1. A DXS spectrum obtained from the dermis of an iron-loaded patient with β-thalassemia major (*solid line*) in comparison to a similar spectrum recorded from a normal control (*dotted line*).

level of Fe and Zn. A study of transfusional iron overload in hemodialysis patients showed also a significant elevation of about 80% in the dermal iron level as measured by DXS.[13] In both studies the DXS values were compared to indirect parameters for iron load, such as serum ferritin levels ($r = 0.6$–0.7) and the number and rate of blood transfusions ($r = 0.6$ and 0.4, respectively). The difference between the iron load in different β-thalassemia patients subjected to a similar rate of blood transfu-

TABLE 1. DXS Measurements of Fe and Zn in the Skin of Normal Controls and in Patients with β-Thalassemia before Chelation Treatment

	Concentration (parts per million)					
	β-Thalassemia Major[b] $n = 70$		β-Thalassemia Intermedia[b] $n = 27$		Normal Controls[b] $n = 20$	
Measurement[a]	Mean ± SD	Range	Mean ± SD	Range	Mean ± SD	Range
Fe, T	32.7 ± 15.7	13.0–120	17.5 ± 11.1	6.7–58.3	11.5 ± 2.4	6.5–13.5
Fe, A	42.0 ± 21.9	13.7–150	18.5 ± 15.3	7.1–74.0	10.2 ± 2.5	5.6–14.0
Zn, T	12.7 ± 2.0	8.6–17.4	13.0 ± 2.7	6.9–18.1	13.4 ± 3.6	7.6–17.6
Zn, A	5.2 ± 1.4	2.9–9.9	5.5 ± 1.4	3.5–8.1	4.5 ± 1.7	3.7–7.4

[a]T, thenar eminence ("epidermal"); A, arm lateral ("dermal").

[b]For β-thalassemia major, mean age of subjects (± SD) was 12.6 ± 5.6 years, with a range of 4–28 years; for β-thalassemia intermedia, 23.1 ± 8.6 years, with a range of 7.5–44 years; for normal controls, 31.8 ± 6.9 years, with a range of 16.5–62 years.

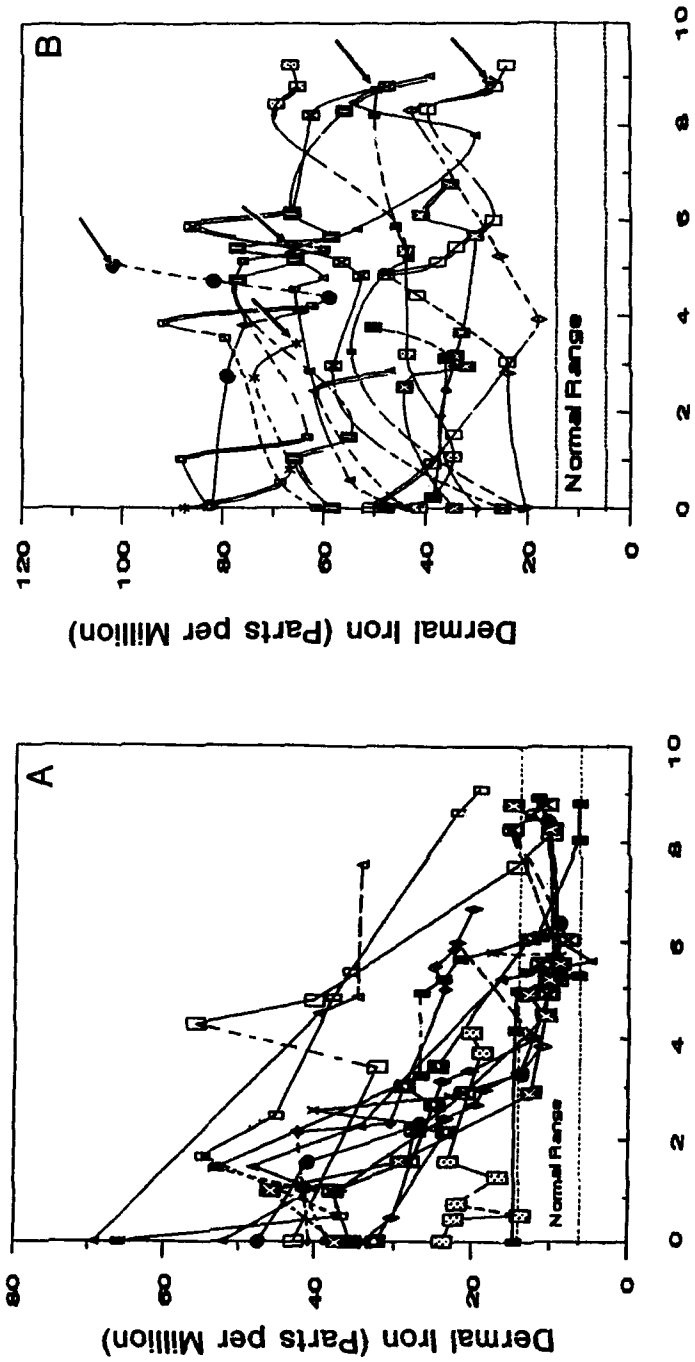

FIGURE 2. A prolonged follow-up of dermal Fe levels in β-thalassemia major during subcutaneous Desferal (DFO) treatment. Twenty-eight patients participating in this long follow-up study were divided into two major groups according to their compliance to treatment. Each *curve* represents the dermal Fe level of a single patient as a function of time. (**A**) Patients with moderate-to-high compliance consuming at least 5 g DFO/week. Regular treatment is denoted by *solid lines*. *Broken lines* represent periods with no compliance as manifested by drastically reduced treatment. (**B**) Patients with low compliance or receiving irregular DFO treatment with an average dose of less than 5 g/week (*solid line*) or with no DFO treatment (*broken line*). The short periods in which the patients received adequate treatment or were hospitalized regularly for intensive intravenous treatment are represented by *double line*. Five patients in this group who died during the follow-up period are identified by *arrows*.

sions probably derives from the specific rate of iron accumulation in each patient, which does not necessarily depend only on the rate of transfusion.

A summary of the data on Fe and Zn skin levels in ninety-seven thalassemia patients (70 major and 27 intermedia) is presented in TABLE 1. Most of these patients had not been subjected to regular chelation treatment at the time of their first examination. As previously reported for a smaller group of patients,[11] both epidermal and dermal Fe was elevated in β-thalassemia major more than in intermedia, and it reached an averge level in β-thalassemia major which was four-fold higher than in normal controls, with no overlap.

The difference between the epidermal and dermal measurements by DXS is manifested by the consistency in the Zn levels in these areas. The epidermal level of Zn is twice the dermal level in normal subjects examined and does not differ from its level in the skin of β-thalassemic patients, in contrast to the extreme alterations in the Fe levels (TABLE 1, FIG. 1).[11] This contradicts the suggestion that thalassemia may also be associted with Zn deficiency,[14] an hypothesis derived from the study of indirect parameters such as serum Zn, rather than tissue Zn levels.

The benefit of the DXS test seems to be not only in the diagnosis of the severity of the parenchymal siderosis but, more importantly, in the ability to follow up the clearance of Fe in patients undergoing chelation therapy. Non-invasive follow-up of skin Fe levels by DXS seems to serve as a most informative indicator of Fe clearance from non-specific parenchymal organs in patients undergoing chelation treatment. FIGURE 2 presents a prolonged follow-up of dermal iron level in a group of 30 patients with β-thalassemia major who have been subjected to subcutaneous Desferal treatment. About half of the patients had relatively good compliance, except for short periods of reduced average treatment dose (FIG. 2A). In general, all these patients had their iron level gradually reduced. In some patients with prolonged adequate treatment, dermal iron level was reduced to the normal range. The second group of patients, with low compliance, had only short durations of adequate chelation therapy with prolonged intervals of no Desferal consumption or poor and insufficient treatment (FIG. 2B). About a third of these patients died during the long period of follow-up. It is obvious that there is a striking correlation between the quality and dose of chelation treatment and the degree of iron clearance from the skin in both groups. Long chelation treatment did not seem to affect skin Zn levels (data not shown).

The DXS did not fail to detect inappropriate chelation treatment for prolonged periods in any of the patients followed up. This non-invasive, rapid examination of the skin seems to serve as a most informative indicator of iron clearance from non-specific parenchymal organs in patients undergoing chelation treatment.

ACKNOWLEDGMENT

We wish to thank Dr. Hava Hall for her help in summarizing the data base for this study.

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In Vivo Measurement of Hepatic and Cardiac Iron in Cooley's Anemia by Nuclear Resonance Scattering

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Patients with Cooley's anemia develop marked hemosiderosis as a result of the many blood transfusions received and an increased amount of gastrointestinal iron absorption. The hemosiderosis may give rise to organ failure.[1] Parenteral chelation therapy can reduce the iron stores.[2] The serum ferritin level is of value in estimating the total amount of iron accumulated, but it does not identify its presence in specific organs. There is a method of gauging hepatic iron,[3] but none, exclusive of biopsy, for measuring cardiac iron. To do this, a new *in vivo*, non-invasive method for measuring iron, utilizing nuclear resonance scattering (NRS),[4-6] was used at the Brookhaven National Laboratory at Upton, New York. The method is based on the NRS of the gamma radiation from the first nuclear level (847 keV) in the iron. The source of the gamma radiation is provided by the neutron activation of $MnCl_2$ to produce a Mn-56 radionuclide. The 847-keV gamma ray is emitted from the first nuclear iron level following the beta-minus decay of the Mn-56 source. The resonantly scattered activity from the iron in the tissues is monitored by two large, high purity, Germanium detectors. The system was verified by comparison with iron levels of liver biopsies determined by atomic absorption spectroscopy (FIG. 1).

The patients' serum ferritin, hepatic and cardiac NRS measurements are summarized in TABLE 1. Twelve patients, of both sexes, with Cooley's anemia are included in this study. The ages varied from 9 to 34 years. All were transfusion dependent, on subcutaneous Desferal by autosyringe; some received additional periodic intravenous Desferal. Five patients had three studies at yearly-to-biyearly intervals, three had two studies, and four had one, for a total of 25 separate sets of measurements. In those that had liver biopsies, there was good correlation between the iron measured in the biopsy tissue and the NRS hepatic measurement. The cardiac iron level determined by NRS was higher in those patients with high hepatic iron levels as measured by NRS (FIG. 2). In some patients, cardiac iron was below detection levels when the hepatic NRS-determined iron level was low. When the NRS determinations were done periodically, there was evidence of a decrease in iron in some of the patients who were on Desferal. Patients with low-level NRS-determined cardiac iron were asymptomatic. The three patients with the highest cardiac levels of iron

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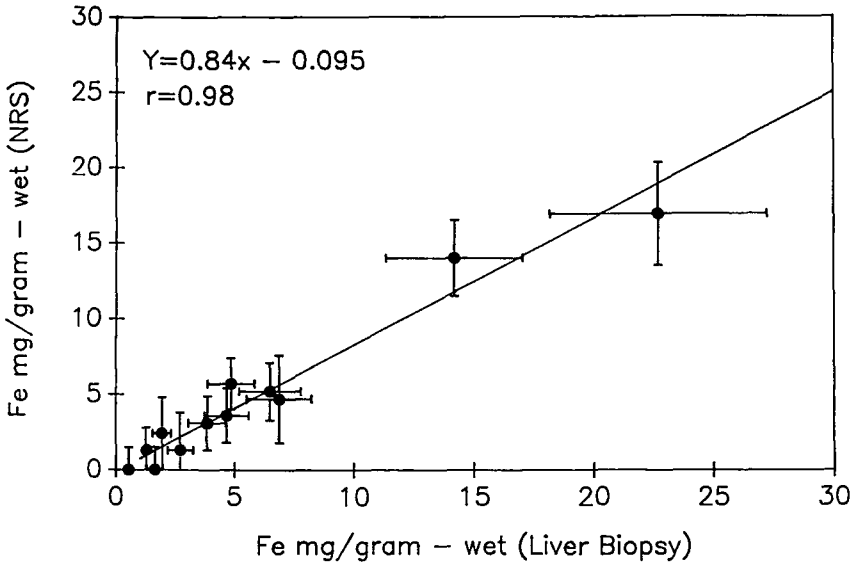

FIGURE 1. Comparison of iron levels determined by atomic absorption spectroscopy of liver biopsy tissue with values obtained by NRS.

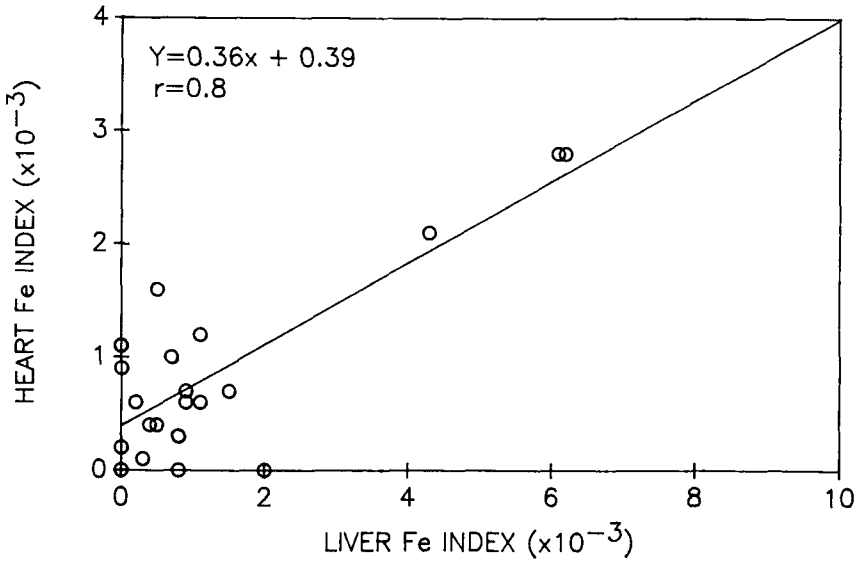

FIGURE 2. Correlation of hepatic and cardiac iron levels measured by NRS.

TABLE 1. Summary of the *In Vivo* Iron Measurements by NRS in Liver and Heart, the Iron Measured in Liver Biopsies, and the Ferritin Levels in Cooley's Anemia Patients

Patient				Liver			Heart	
No.	Sex	Age (yr)	Assay Date	NRS Fe Index ($\times 10^{-3}$)	NRS Fe Conc. (mg/g wet)	Biopsy Fe (mg/g wet)	NRS Fe Index ($\times 10^{-3}$)	Ferritin (mg/ml)
1.	M	19	2/85	6.2 ± 0.8	14.0 ± 2.5	14.2	2.8 ± 0.7	6919
2.	M	21	2/85	4.3 ± 0.9	9.2 ± 2.7	—	2.1 ± 0.6	5660
3.	M	15	6/85	0.8 ± 0.8	2.7 ± 2.7	—	0.3 ± 0.7	496
4.	F	8	9/84	—	—	—	—	1460
			6/85	0.7 ± 0.8	2.1 ± 3.6	—	1.0 ± 0.7	—
			8/86	0	0	—	0	—
			1/90	—	—	—	—	53
5.	F	30	9/85	6.1 ± 0.9	16.9 ± 3.4	—	2.8 ± 0.8	—
			6/86	—	—	22.7	—	—
6.	M	3	6/82	—	—	1.96	—	1068
			2/86	1.1 ± 0.8	2.4 ± 2.4	—	1.2 ± 0.6	913
			2/87	0.2 ± 0.8	0.5 ± 1.7	—	0.6 ± 0.6	—
			6/88	—	—	—	—	2370
7.	M	14	6/85	—	—	0.53	—	—
			2/86	0	0	—	1.1 ± 0.8	1791
			12/86	1.5 ± 0.9	3.1 ± 1.8	—	0.7 ± 0.7	3810
8.	F	31	9/85	0.8 ± 0.8	—	—	0	835
			11/86	0	0	—	0.9 ± 0.8	—
			11/88	0	0	—	1.1 ± 1.0	—
9.	F	8	8/85	0.5 ± 0.7	1.3 ± 2.5	1.28	1.6 ± 0.6	700
			11/86	0.3 ± 0.6	0.8 ± 1.8	—	0.1 ± 0.4	512
			11/88	0.9 ± 0.7	2.6 ± 2.1	—	0.6 ± 0.5	264
10.	M	10	8/85	0	0	—	0	—
			12/86	0.9 ± 0.8	2.2 ± 1.9	—	0.7 ± 0.4	3702
			6/87	—	—	—	—	4172
			11/88	1.1 ± 0.8	2.5 ± 1.8	—	0.5 ± 0.4	5840
			6/89	—	—	—	—	5960
11.	M	10	4/85	0	0	—	0	130
			12/86	0.8 ± 0.8	2.0 ± 2.0	—	0.3 ± 0.6	253
			12/88	0.4 ± 0.9	1.0 ± 2.0	—	0.4 ± 0.6	303
			6/89	—	—	—	—	230
			1/90	—	—	—	—	53
12.	M	12	6/81	—	—	6.87	—	7600
			4/85	2.0 ± 0.9	4.7 ± 2.9	—	0	4740
			8/86	0.5 ± 0.8	1.2 ± 1.8	—	0.4 ± 0.5	2148
			12/88	0	0	—	0.2 ± 0.8	1270
			6/89	—	—	—	—	1180

measured by NRS were symptomatic with cardiac dysfunctions, such as tachyarrhythmia and congestive heart failure, fatal in two patients. Postmortem examination done on one of the two patients showed marked cardiac and hepatic hemosiderosis. Other patients, with repeat NRS determinations, showed a decrease in hepatic iron and an increase in cardiac iron, possibly a result of redistribution of iron with the chelation.

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A New System for Subcutaneous Infusion of Desferrioxamine

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Iron-loaded patients need daily iron chelating therapy by slow, subcutaneous administration of desferrioxamine.[1] This is generally carried out overnight at home using a portable infusion pump.[2] Different types of pumps are available, albeit all mechanical with battery-powered electric engines. Weights range from 250 to 600 g. There are frequent difficulties with using mechanical pumps: battery failure, forgetting to switch the engine on, damaging the pump by enuresis. Psychological problems due to the discomfort of wearing a heavy and bulky infusor are also common.[3]

Due to these reasons, high compliance with this therapy is hard to achieve, and many patients do not succeed in reducing their iron overload. A new system for subcutaneous infusion has been tested and compared to the traditional one.

The system consists of a gas-producing cell, a head, and a special 25-ml syringe (Disetronic Ltd., Switzerland). The syringe must be filled with desferrioxamine solution, then the cannula of the plunger has to be removed. Finally, after the gas cell is introduced into the head, the head must be screwed to the back of the syringe. At this juncture the setting of the pump is complete and the infusion starts, as the gas (hydrogen) coming out of the cell pushes the plunger.

The whole infusor set weighs 54 g; its overall dimensions are 14.5 cm (length) by 2.5–4 cm (diameter). The new infusor set is compared to a traditional one in TABLE 1.

In 15 regularly transfused β-thalassemic patients, 161 infusions were performed with the new system; and these data were compared with those obtained in the previous year using the traditional mechanical pump (with the same desferrioxamine doses for each patient in a 10-ml volume). Patients' ages ranged from 6 to 18 years, desferrioxamine dose between 0.5 and 2.5 grams.

During each infusion the flow velocity was perfectly constant. The mean flow velocity rate was 2.20 ± 0.21 ml/h. There was no correlation between flow velocity and desferrioxamine concentration in the solution. The mean infusion time was

TABLE 1. Comparison between the New Gas Cell Infusor and a Traditional Mechanical Infusor

Property	Gas Cell Infusor	Traditional Infusor
Weight (g)	54	240
Overall dimensions	14.5 cm long, 2.5–4 cm wide	$17 \times 7 \times 2$ cm^3
Syringe volume (ml)	25	10
Mean infusion time (h)	11.45 ± 1.12	10.3 ± 1.02
Failure rate (%)	7.8	5
Local side effects	No	Yes (22.7%)

11.45 ± 1.12 h (10.3 ± 1.02 h with the traditional pump). Failure rate, i.e., an infusion time > 14 h, was 7.8% (5% with the mechanical pump). No local side effects were recorded. In all cases, at the end of each infusion a very mild edema without inflammation was present, but it disappeared within 1–2 h. With the standard infusors the prevalence of local side effects, mainly subcutaneous nodules, was 22.7%.

On the one hand this new system seems to have certain advantages: (1) From the practical point of view, preparation procedures are simpler and quicker. Problems related to the use of a mechanical device—the need for power, for switching it on, for adjustments, and for setting the pump syringe—are avoided. (2) The whole system can be considered safer. All parts except the head are disposable, and the complete set is waterproof. (3) There is no pulsatility in the infusion of the drug, as there is in most traditional pumps.

On the other hand, the system can be improved: (1) The failure rate is acceptable, but it is still higher than that observed using mechanical pumps. This seems to be due to the gas cells we utilized. They were the first set produced specifically for the study, and they are not yet standardized. Automated production is now in progress, and we think that it will ensure a good and constant quality for the gas cells. (2) The 25-ml syringe volume was experimental too. We are going to perform tests with 10-ml and 20-ml syringes, to ascertain whether the difference in the rate of local side effects is due to the different concentrations of the drug injected.

Above all, the preliminary data are encouraging and we suppose that the use of a gas-producing infusor could lead to higher compliance, that is to say greater subcutaneous chelating therapy efficacy.

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Use of Central Venous Access for Iron Chelation Therapy[a]

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Our chronic transfusion patients are treated with home Desferal (DF) 5–7 times/week and, in addition, pulse high-dose intravenous (IV) DF for 24–48 h at the time of each transfusion to chelate iron released from nonviable donor red cells. This IV DF therapy therapy can chelate 30–50% of the transfusional iron.[1] Ten patients required central venous access (CVA). TABLE 1 summarizes their ages, diagnoses, types and

TABLE 1. Clinical Data for Patients Requiring CVA

Patients (*n*)	10
Age (yr)	
Range	4–36
Median	13
Diagnosis (*n*)	
Thalassemia	5
Sickle cell anemia	2
Other anemias	3
Type of CVA (*n*)	
Port	8
Hickman	1
Both	1
Primary indication (*n*)	
Venous access	7
Chelation therapy	3
Duration (yr)	
Range	<0.6 to >5.5
Median	1.5
IV DF infusions through CVA (*n*)	
In hospital	1173
Home	4104
Total	5277
Complications[a]	
Requiring removal/replacement (*n*)	
Sepsis	5
Clotting	3
Potential for acute hypotension or anaphylaxis	No severe problems to date[b]

[a]**Precautions:** Have second person available when starting or discontinuing CVA. Flush CVA *slowly* to avoid bolus infusion of DF. Use micropore filter with IV DF. Provide anaphylaxis kit and instructions.

[b]One patient without CVA had an episode of hypotension from a bolus infusion of a solution containing DF precipitates immediately after starting IV DF using a *peripheral vein*.

[a]This study was supported by the Italian Catholic Federation and other Italian/American organizations.

TABLE 2. Desferal (DF) Therapy by CVA

Patient No.	Age (yr)	Intravenous Desferal: Home mg/kg/8–16 h	Home Infusions (n)	Hospital mg/kg/24 h	Hospital Infusions (n)	Total Infusions (n)	Ferritin (ng/ml) Before	After	% Change	DF Toxicity	Comments
1.	4	—	0	275	63	63	421	1880	↑78	0	
2.	10	69	12	234	134	146	1529	2304	↑34	0	
3.	13	89	112	—	—	>112	1810	1668	↓8	0	
4.	17	45–90	426	90–269	129	555	1507	239	↓84	0	DF therapy modified according to serum ferritin.
5.	36	63	30	—	—	>30	1147	1439	↑21	0	Subcutaneous DF not tolerated.
6.	10	—	0	334	152	152	6916	4304	↓38	0	
7.	31	57	65	—	—	>65	11625	8454	↓28	0	Cardiac symptoms.
8.	7	0–55–110	1812	0–110–165	229	2041	7132	4380	↓39	Sensorineural hearing loss[a]	Transfusion requirement: 50–70/yr since age 2 yr. No cardiac problems or diabetes. General condition good.
9.	13	70	685	290	325	1010	2423	1678	↓31	0	
10.	34	49–98–147	962	293	141	1103	2400	300	↓87	0	Progressive severe cardiac arrhythmias and diabetes. Striking clinical improvement. Still requires medications. Married, works full time. DF therapy modified according to serum ferritin.

[a]Hearing improved after DF therapy modified.

indications for CVA, number of DF infusions and complications. TABLE 2 details each patient's DF dose and number of infusions in the home and hospital, as well as the effect of DF on serum ferritin levels, DF toxicity and additional information about the patients.

Before modification, the DF dose in the hospital, 165–334 (median, 275) mg/kg/24–48 h, was much higher than the home dose of 57–147 (median, 80) mg/kg/8–16 h, 5–7 times/week. Serum ferritin levels decreased to ≤ 300 ng/ml in two patients, necessitating less aggressive chelation. In seven others, serum ferritin levels stabilized with less than 50% change in either direction. In one patient, ineligible for home chelation, serum ferritin increased by 78%. There were no DF-related problems at the time of infusion, other than occasional mild gastrointestinal symptoms. Sensorineural hearing loss occurred in one patient and improved with reduced DF therapy. Eye examinations have not shown retinal damage or cataracts. Sepsis or clotting necessitated removal of the CVA eight times in >21 patient-years. An episode of nonfatal severe hypotension occurred at the start of an IV DF infusion of a solution containing DF precipitates in one patient not in the study because he does not have CVA. This happened after 10 years of chelation therapy.

After 5.5 years and more than 5,000 DF infusions via CVA, we find that IV DF is safe in the hospital and for selected patients in the home, provided there is preparation of patient/parent for possible anaphylaxis. Compliance with home IV DF is excellent and better than with subcutaneous DF. CVA enables effective chelation for patients with life-threatening cardiac or other severe iron-related problems for whom subcutaneous DF is inadequate. It also facilitates transfusion and DF therapy for patients with severe venous access problems, and it may provide an acceptable alternative for home DF treatment in some patients with poor compliance with subcutaneous DF because of local discomfort. For these latter patients, home IV DF may prevent the cardiac and other complications of iron overload which would otherwise be inevitable. Although CVA decreases hospitalization time and cost, this is not an indication for placement.

Despite treatment with DF doses of 165–334 mg/kg/24/h in 7 patients, only one developed moderate sensorineural hearing loss, and no patients had eye problems. The availability of chelatable iron from nonviable donor red cells at the time of these infusions and the fact that the maximum duration per treatment is only 48 h may explain the low toxicity.

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The Interference of Pyridoxal Isonicotinoyl Hydrazone with Intestinal Iron Absorption[a]

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A number of new oral iron-chelating compounds have been developed for the treatment of iron overload. Among these, acute and subacute toxicity tests suggest that pyridoxal isonicotinoyl hydrazone (PIH) has very low toxicity in both animals and humans.[1] Phase II clinical trials are now being conducted. Although some chelators showed high efficiency in iron excretion, long-term oral administration at low dose failed to show any reduction in hepatic iron in animals.[2] It has been proposed that the "low-dose effect" is due to their capacity to transport dietary iron across the gastrointestinal (GI) barrier. The aim of this study is to determine whether PIH has any enhancing effect on intestinal iron absorption, a criterion for further clinical evaluation.

Studies were performed in 6 adult male patients, aged 15–31 years, with β-thalassemia/Hb E disease who were not regularly transfused. All were in steady-state without complication or recent blood transfusion at the time of study. They were markedly anemic with moderate degrees of iron overload. No medication was given three days before and during the four-week study period except as described below.

Two iron absorption measurements were performed in each patient by the whole-body counting technique,[3] one with oral PIH plus radioiron ascorbate, the other as a control of radioiron absorption. To prevent acid hydrolysis of PIH, 150 mg of ranitidine was given twice a day, at 6 A.M. and 6 P.M., for five days (days −2, 0, +2). Overnight-fasted subjects were fed with 1 μCi (5 mg) [^{59}Fe]ferrous sulfate with 50 mg ascorbic acid plus 3 capsules of 200 mg PIH. The percent radioiron retention was evaluated on day 14, when the same dose of radioiron ascorbate was readministered; and iron absorption was measured on day 28 as the control. Hematologic examina-

[a]This work was supported by U.S. Public Health Research Grants HL 34408 and HL 42814-01 from the National Heart, Lung and Blood Institute.

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TABLE 1. Hematologic Examination, Iron Status, and Iron Absorption Studies[a]

Subject	Hb (g/dl)	Retic (%)	SI (μmol/l)	TIBC (μmol/l)	Tf Sat (%)	SF (ng/ml)	Fe Absorption (%) Control	Fe Absorption (%) PIH-treated
1	5.8	9.0	31.7	33.4	95	1928	53.4	45
2	5.9	12.5	47.4	42.6	111	4453	61.8	24.2
3	6.4	10.1	51.9	57.9	90	1981	52.2	18.4
4	7.8	35.4	31.7	41.6	76	1321	63.2	12.2
5	6.3	26.0	41.1	41.4	99	1712	67.5	27.7
6	6.6	32.3	48.3	46.6	104	1482	65.7	45.5
Mean	6.5	20.9	42	43.9	96	2146	60.6	28.8
± SD	± 0.7	± 11.8	± 8.7	± 8.1	± 12	± 1158	± 6.4	± 13.8

[a]Hb, hemoglobin; Retic, reticulocytes; SI, serum iron; TIBC, total iron binding capacity; Tf Sat, transferrin iron saturation; SF, serum ferritin.

tion and iron status measurement were performed using standard ICSH (International Committee for Standardization in Haematology) methods.

The patients were severely anemic, serum iron values were increased, transferrin iron saturation was complete, and serum ferritin levels were elevated (TABLE 1). Control iron absorption values surprisingly ranged from 52.2 to 67.5%. PIH at a dosage of 600 mg significantly inhibited GI iron absorption (FIGURE 1), the mean value decreasing from 60.6 ± 6.4% to 28.8 ± 13.8% (TABLE 1).

In these β-thalassemia/Hb E disease patients, iron overload is due to inappropriate increased absorption of dietary iron through the intestinal mucosa. Low-dose oral PIH inhibited this excessive absorption significantly although it is only a modest iron chelator. This effect, together with its high safety margin and moderate cost,

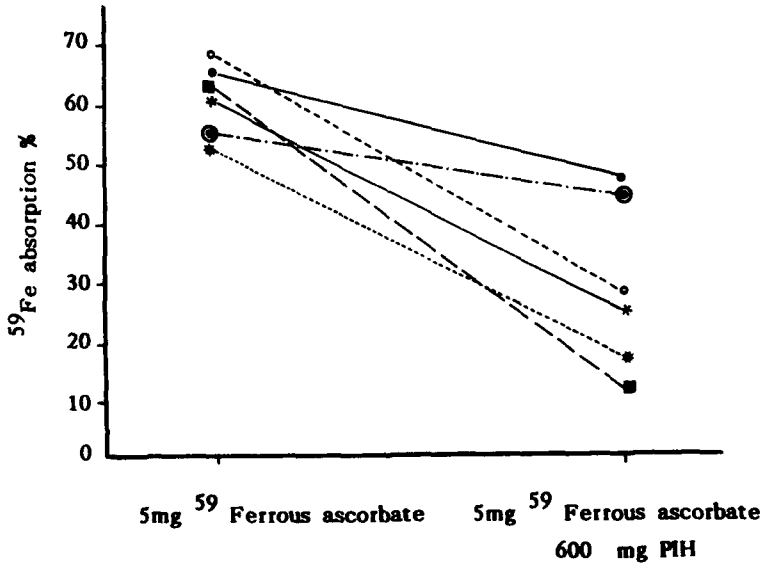

FIGURE 1. Effect of PIH (600 mg) on GI iron absorption in six patients.

should encourage further clinical trials of PIH for the eventual purpose of preventing development of hemochromatosis in young thalassemic children or further iron accumulation in adult patients.

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Cardiac Disease–free Survival in Patients with Thalassemia Major Treated with Subcutaneous Deferoxamine

An Update of the Toronto Cohort

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Previously we reported cardiac disease–free survival in transfused thalassemia major patients begun on subcutaneous deferoxamine (DFO) after the age of ten years.[1] There were 36 patients included in this analysis (23 from the Toronto cohort, 13 followed at Boston Children's Hospital) of whom, in 1985, eight had died of iron-related cardiac disease. Of these eight patients, one had been considered compliant with nightly subcutaneous DFO, and seven were considered non-compliant with this regimen. Five years later, a re-examination of the Toronto cohort by more detailed cardiac testing and a review of compliance demonstrates that even within the group of patients presumed "compliant" with DFO, a high incidence of cardiac disease has now been observed. Of the 11 Toronto patients in the original "compliant" group, 3 have died of cardiac disease, 4 are presently maintained on digoxin and/or anti-arrythmic agents, and 1 patients has heavy cardiac iron loading, as demonstrated by magnetic resonance imaging, and diastolic dysfunction, as demonstrated by exercise radionuclide scanning (MUGA). Of the 12 "non-compliant" Toronto patients, 7 are dead and 4 are maintained on cardiac medications.

We then examined the outcome of all 37 Toronto patients with thalassemia major begun on DFO after the age of 10 years (Group 1), including 14 who were not included in the earlier analysis. The mean age of this group at the start of DFO therapy was 15.4 years; the mean age at present (in 25 living patients) is 23.9 years. In this group, 12 patients have died of iron-related cardiac disease (CD), and 9 of the 25 surviving patients have iron-related CD requiring digoxin and anti-arrythmic agents. The remaining 16 patients are alive and require no cardiac medications. In the patients with normal clinical cardiac function and normal systolic function by echocardiogram, diastolic dysfunction by MUGA is present in 68%. If clinical CD alone is considered, cardiac disease–free survival in Group 1 is 43%.

We next examined the cardiac disease–free survival in a group of 17 patients with thalassemia major begun before the age of 10 years on subcutaneous DFO (Group 2), who are now at a mean age of 17 years, allowing meaningful comparison to the older group for the outcome of iron-related CD. Of this group of patients, whose

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mean age at the start of DFO therapy was 7,6 years, all patients are alive, and only one patient has medication-dependent CD. In patients in Group 2 with normal cardiac function by clinical history and systolic function studies, 2 patients (12%) have diastolic dysfunction by MUGA. Considering clinical CD alone, the cardiac disease–free survival in Group is 94%.

When re-analysis of compliance with DFO was carried out, a significant decline in the percentage of the prescribed drug which was actually administered was noted from age 10 to 20 years. Group 2 patients had consumed significantly more DFO than had Group 1 patients, despite an equal time on therapy (mean ± SD: 9.3 ± 1.5 years, Group 1; 9.5 ±1.0 years, Group 2). The level of prechelation serum ferritin (SF) and the number of years in which SF was elevated above 2000 μg/l were also significantly different between Groups 1 and 2 ($p < 0.01$ and $p < 0.0005$, respectively).

This analysis suggests that even those patients with thalassemia major who do comply with DFO treatment may not enjoy cardiac disease-free survival if begun on therapy after 10 years of age. However, it appears that the group of patients who are now at an age at which CD would be expected, and who were begun on DFO before the age of 10 years, have preserved cardiac function, even as assessed by sensitive parameters of testing.

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