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The Thalassemias: The Role of Molecular Genetics in an Evolving Global Health Problem*

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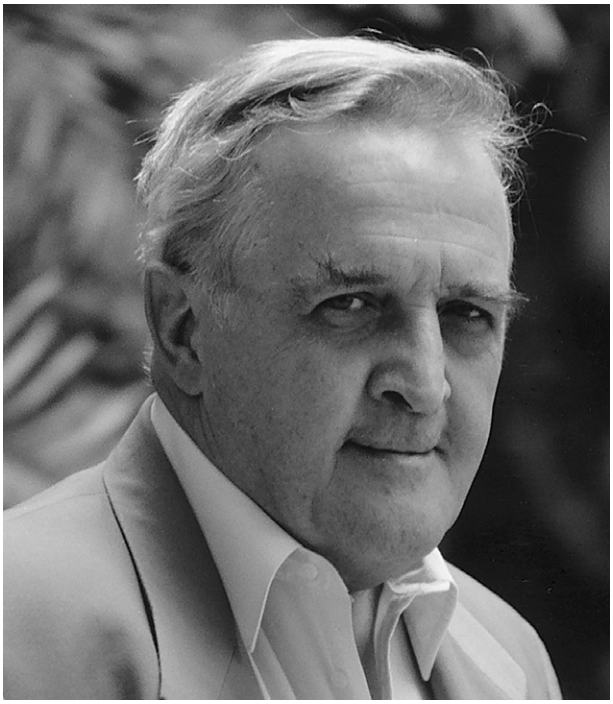

David Weatherall

Because the thalassemia field has never been in the mainstream of human genetics, and hence its contribution to a better understanding of the molecular pathology and cellular basis for the phenotypic diversity of monogenic diseases is not widely known, it is a particular pleasure to accept this award on behalf of my many friends and colleagues of diverse disciplines who have worked in it over many years.

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The Development of the Thalassemia Field

Although it is now realized that the thalassemias are the commonest monogenic diseases in humans, this was not always the case. The early history of thalassemia is described in detail elsewhere (Weatherall and Clegg 2001). It was first recognized as a distinct entity independently by workers in the United States and Italy in 1925. It received its name, derived from Greek roots meaning "sea" and "blood," in the mistaken belief that it was restricted to individuals of Mediterranean background. However it gradually became apparent that it is a disease of widespread occurrence in populations ranging from Africa and the Mediterranean region, through the Middle Eastern and Indian subcontinent, to Southern China and Southeast Asia. Work in the 1940s, again carried out independently in the U.S.A. and Italy, showed that the disease is inherited in a Mendelian recessive fashion. And, by this time, it was already clear that the thalassemias are characterized by a wide range of clinical disability and that there might be more than one form of the condition. But, until the 1950s, there was no understanding of the underlying cause.

During the 1950s, there was rapid progress toward an understanding of the structure and function of human hemoglobin. It was found that normal adults have a major hemoglobin (Hb), called "Hb A," comprising about 90% of the total, and a minor component, "Hb A_2," which accounts for 2%–3%. The main hemoglobin in fetal life is Hb F, only traces of which are found in normal adults. Earlier during development, there are three different embryonic hemoglobins. All these different hemoglobins were found to be tetramers of two pairs of unlike globin chains; adult and fetal hemoglobins have α chains combined with β (Hb A, $\alpha_2\beta_2$), δ (Hb A_2, $\alpha_2\delta_2$), or γ chains (Hb F, $\alpha_2\gamma_2$). In embryonic life, α-like chains, called "ζ chains," and β-like chains, called "ε chains," combine with γ or α chains to produce the different embryonic hemoglobins. The amino acid sequence of these different globin chains was determined, considerable progress was made toward an understanding of the three-dimensional structure of hemoglobin, and its allosteric properties as an oxygen transporter were explored.

Following Pauling's observation that patients with sickle-cell anemia have an abnormal hemoglobin (Pauling et al. 1949), scientists in many parts of the world started to analyze the electrophoretic pattern of human hemoglobins and found many abnormal variants. These might affect either the α- or β-globin chains; Hb S, for example, was found to be a β-chain variant. At the same time, several seminal observations were made that set the scene for the further understanding of the thalassemias. It was found that many patients with thalassemia have persistent fetal hemoglobin after birth and that most carriers have elevated levels of Hb A_2. Of particular importance was the observation that those who inherit the sickle-cell gene from one parent and thalassemia from the other have a completely different pattern of hemoglobin production from those with the sickle-cell trait; doubly affected persons show a higher level of Hb S than Hb A, the opposite of the situation that occurs in the sickle-cell trait. This suggested that the action of the thalassemia gene might be to reduce the level of production of β chains of human hemoglobin. At the same time, patients with the clinical picture of thalassemia were discovered who had abnormal hemoglobins consisting of only β chains, β_4 tetramers, designated "Hb H."

These diverse observations were integrated in 1959 to develop a hypothesis about the possible genetic basis for the thalassemias. It was suggested that those that interact with β globin variants like Hb S affect the production of β chains of hemoglobin, while those that are associated with β-chain homotetramers might affect the production of the α chains of hemoglobin. In short, there might be two main types of thalassemia, α and β (Ingram and Stretton 1959). And another important observation was made at about this time. It was found that the red-cell precursors of patients with β thalassemia contain ragged inclusion bodies (Fessas 1963). Could these be an abnormal and unstable hemoglobin due to a mutation of the β-globin genes that had hitherto been overlooked, were they excess α chains that had accumulated due to defective β-chain production, or was there an even more subtle defect in the association of the globin chains in this condition? Clearly, an approach was needed to try to explain all these disparate observations and put the field on a harder experimental footing.

In the 1960s, a method was developed for measuring the in vitro synthesis globin chains in a quantitative fashion (Clegg et al. 1965; Weatherall et al. 1965). It was found that both the α and β thalassemias are characterized by imbalanced globin-chain synthesis. In some forms of β thalassemia, no β globin chains are synthesized, while in others they are produced at a reduced rate. The excess α chains consequent on defective β-chain production are highly unstable and rapidly precipitate to become associated with the red-cell membrane (Bank and Marks 1966). On the other hand, in the moderately severe forms of α thalassemia—Hb H disease, for example—the excess β chains form a large intracellular pool, some of which are available to combine with β chains to form Hb A, while the others form Hb H (Clegg and Weatherall 1967). In the more-severe forms of α thalassemia associated with fetal death and hydrops fetalis, no α chains are produced at all (Weatherall et al. 1970). These experiments were extended to analyze the rates of initiation and elongation of the globin chains in thalassemic patients. It was found that both these mechanisms are normal in patients with α and β thalassemia (Clegg et al. 1968; Weatherall and Clegg 1969). Thus, it appeared that the most likely cause of defective α- or β-chain production lay in a reduced amount of messenger RNA for the affected chains, at least in the forms of thalassemia that were studied in this way. Complementary to this work on globin synthesis, careful studies of the turnover of the different hemoglobins in patients with thalassemia, compared with a detailed analysis of their distribution together with that of inclusion bodies in the red cells, established a better understanding of the pathophysiology of the disease (Nathan and Gunn 1966).

Although, until the mid-1970s, it was not possible to analyze globin genes directly, some progress was made toward an understanding of what might be happening at the DNA level. Using either rabbit-reticulocyte-lysate or mouse-ascites-tumor systems, together with in vitro globin synthesis, it was found that there is a deficiency of functional β-globin messenger RNA in β-thalassemic red cells (Benz and Forget 1971; Nienhuis and Anderson 1971). It was also found that some forms of thalassemia are associated with the production of abnormal hemoglobins. For example, some patients with the clinical picture of β thalassemia were found to have an abnormal hemoglobin, called "Hb Lepore" after the family name of the first patient in whom it was discovered, which consisted of α chains combined with variant chains that were part β and part δ. It was suggested that the latter had arisen by abnormal crossing over between the β and δ globin genes, with the production of ineffectively synthesized $\delta\beta$ fusion products (Baglioni 1965). And some patients with α thalassemia were found to have traces of a slowly migrating hemoglobin that was also produced at very low levels and, in this case, was found to consist of a variant α chain that was elongated at its C terminal end. It was suggested that this resulted from a mutation in the α-chain termination codon, with read through of messenger RNA that is not normally translated (Weatherall and Clegg 1975). This hypothesis was validated later when it was found that the amino acid composition of the elongated portion of the α chain was

completely consistent with the base sequence of the normally untranslated region of α globin messenger RNA.

Careful genetic studies of the α thalassemias suggested that there might be two main forms, α° thalassemia and α^{+} thalassemia, in which there was a total or reduced rate of α-chain production, respectively (Pootrakul et al. 1967; Na-Nakorn and Wasi 1970). In the mid-1970s, using cDNA/DNA hybridization, it was found that, in homozygotes for α° thalassemia, the α globin genes are deleted, the first clear demonstration of a deletion in a human monogenic disease (Ottolenghi et al. 1974; Taylor et al. 1974). And structural analyses showed that some patients with β° thalassemia—that is, those who make no β chains whatever—produce full-length β-globin mRNA (Old et al. 1978); the first example of a human nonsense mutation was discovered by a similar approach (Chang and Kan 1979).

By the end of the 1970s, it became possible to clone and sequence the globin genes; there were few surprises, and the order that had been predicted by simple family studies over the previous 20 years proved to be correct. The application of Southern blotting, together with the development of the rapid DNA-sequencing methods, led to extensive studies of the molecular pathology of the disease, work that was facilitated by the discovery of the strong association between different mutations of the α- and β-globin genes with restriction fragment–length polymorphisms of the α- and β-globin gene clusters (Orkin et al. 1982; Higgs et al. 1986). It is now known that there are over 200 different β-globin gene mutations that underlie β thalassemia and a large number of different deletions and nondeletional forms of α thalassemia (Weatherall and Clegg 2001).

These advances in knowledge of the pathophysiology and molecular pathology of the thalassemias led to considerable progress in their control and management. Carriers are relatively easy to identify and—using, first, globin-chain synthesis and, later, DNA technology—screening and prenatal diagnosis programs were established in many countries, leading to a dramatic reduction in the frequency of new births of children with severe forms of thalassemia in some of the Mediterranean islands and mainland populations and to considerable progress in this direction in a number of other countries (Weatherall and Clegg 2001). The use of adequate transfusion regimens combined with iron-chelation agents have altered the prognosis for many severely affected β thalassemic patients (Brittenham et al. 1994; Olivieri et al. 1994), although, because of the cost and difficulties in compliance, many children are still dying with this condition early in life in the developing countries. Bone-marrow transplantation has been applied successfully in patients with HLA-matching donors (Thomas et al. 1982; Giardini 1997), but attempts to treat the condition more directly, either by elevating the level of fetal hemoglobin or by somatic cell gene therapy, have not yet been successful.

Current and Future Problems for the Control and Management of the Thalassemias

Phenotype-Genotype Relationships

Although probably more is known about the genetic basis for the remarkable variation in clinical phenotype observed in the thalassemias than for any other monogenic disease, many problems remain.

In the case of the β thalassemias, the multilayered complexity of the genetic basis for phenotypic diversity is best explained in terms of primary, secondary, and tertiary genetic modifiers (Weatherall 2001). The primary modifiers represent the broad diversity of mutations that affect the β globin genes, ranging from extremely mild promoter mutations that cause a very slight reduction in β globin–chain production to the many different mutations that result in the β° thalassemias; that is, a complete absence of β-globin product. Compound heterozygosity for these different mutations can provide a very broad spectrum of clinical phenotypes. The secondary genetic modifiers are those that are involved directly in modifying the degree of globin-chain imbalance in β thalassemia. The coinheritance of α thalassemia has this effect, and, since there are numerous different molecular forms of α thalassemia of different severity, this interaction provides further scope for a wide range of different β thalassemia phenotypes. Similarly, the degree of globin-chain imbalance can be reduced by the more effective synthesis of the γ chains of fetal hemoglobin after birth. It is now known that there are several genes involved in modifying the γ-chain response, some that are encoded in the β globin–gene cluster, others that are on different chromosomes. The tertiary modifiers, those that are not related to globin chain production but that may have an important effect on the complications of the disease, include genes involved with iron absorption, bilirubin metabolism, bone metabolism, and, probably, susceptibility to infection.

As in the case of most monogenic diseases, the effect of the environment has, until recently, been neglected as a potential phenotypic modifier. However, ongoing studies in Sri Lanka suggest that malaria may play an important role, an observation that, if confirmed, is of considerable importance in light of the recrudescence of malaria as a major killer in many developing countries.

Many questions remain, however. There is very little information about the interactions of different genetic modifiers, clear evidence that other modifiers remain to be discovered, and at least a hint that the clinical phenotype of the thalassemias, which changes during differ-

ent stages of development, may reflect innate variation in the ability to adapt to severe anemia.

Population Genetics and Dynamics

Although J. B. S. Haldane originally suggested in 1949 that the high frequency of the thalassemias might reflect heterozygote advantage against severe malaria (Haldane 1949), it is only recently that definite evidence has been obtained that his hypothesis was correct. It has been found that, in the case of the milder forms of α thalassemia, homozygotes—that is, those with two out of four α genes ($-\alpha/-\alpha$)—and probably heterozygotes ($-\alpha/\alpha\alpha$) are resistant to the complications of *Plasmodium falciparum* malaria. Remarkably—at least in Papua New Guinea, where these studies were carried out—those with α thalassemia are also more resistant to other childhood infections, notably upper-respiratory-tract infection (Allen et al. 1997). Whether this is mediated through their better overall health due to protection against the debilitating effects of chronic malaria or whether it is due to a more direct effect of α thalassemia on their immune status remains to be determined. But whatever the mechanism, it is clear that the α thalassemias are having a major effect on the overall health of children in highly malarious regions. Although the appropriate case-control studies have not yet been completed, there is strong circumstantial evidence that the high frequencies of β thalassemia and Hb E may also reflect heterozygote resistance to malaria.

Since it is now clear that many genetic polymorphisms other than those involving hemoglobin have been maintained by differential susceptibility to malaria (Weatherall and Clegg 2002), and because, in evolutionary terms, human exposure to malaria is fairly recent, it follows that patients with thalassemia from different parts of the world are likely to have different genetic constitutions with regard to susceptibility to malaria and a wide range of other infectious organisms. Further work in this field should tell us a great deal about how natural protection against malaria is mediated, to what extent genetic resistance needs to be taken into account in the evaluation of potential malaria vaccines, and some of the reasons for the wide range and patterns of susceptibility to infection that are shown by patients with severe forms of thalassemia, a phenomenon that has never been adequately explained. In view of the recrudescence of malaria as a major health problem in many countries, these questions are likely to be of importance for some time to come.

Clinical Management

Although the introduction of bone-marrow transplantation has offered the possibility of curing severe forms of thalassemia, particularly if carried out early in life, this is available only to a relatively small number of patients in the developed countries and to an even smaller fraction of those in the developing world. The position will only be improved if the methods become available for bone-marrow transplantation between nonmatching individuals or with the development of somatic-cell gene therapy. Both these developments may take a long time to come to fruition and will probably be extremely expensive. It is equally important, therefore, to continue research into better methods for elevating Hb F levels, particularly in the case of intermediate forms of β thalassemia, which may be improved very considerably by a small incremental rise in the steady-state hemoglobin level (Olivieri et al. 1997).

However, regular treatment with blood transfusion and iron-chelating agents, if pursued vigorously, is allowing children with serious forms of β thalassemia to survive into adult life in good health. The major problems to be resolved are the delivery of this type of care to the poorer countries of the world, difficulties with compliance, and, in particular, the lack of an oral chelating agent of proven effectiveness.

Population Control

It is still not widely appreciated that the thalassemias are posing an increasingly serious health burden in many parts of the world, particularly the countries of Asia. For example, it has been assessed recently that there are between half and three quarters of a million severely affected children in Thailand (World Health Organization [WHO] 2002) and that the gene frequency in Indonesia is such that to treat even a proportion of patients in the future will require between 1.25 and 1.5 million units of blood each year (de Silva et al. 2000). The major challenge for the developing countries is, therefore, to evolve programs for the population control of the thalassemias. This requires widespread public education, the development of screening and counseling programs, the establishment of at least one or two central laboratories for the more definitive diagnosis of these conditions, training clinicians to develop expertise in management, and a decision on the part of the population as to whether it wishes to develop prenatal diagnosis programs. Considering the extensive cultural and religious differences in attitude to prenatal diagnosis, the latter approach to population control requires a major effort at education and long discussions between the communities and well-informed doctors.

While there are many excellent examples of national thalassemia-control programs, notably those in Cyprus and Sardinia (Cao 1994; Angastiniotis and Modell 1998), many problems remain. In some countries, there are cultural or religious objections to the termination of pregnancy, although the views are slowly changing. These

programs should not be introduced quickly into societies where there are considerable doubts about the ethical issues involved. Rather, there should be adequate education programs and counseling facilities available and the population should, by a gradual process of debate and social evolution, decide whether they wish to move toward the type of control programs that have been so successful in the Mediterranean countries. Currently, a number of countries in the developing world are moving in this direction. However, because control programs of this type are undoubtedly cost effective compared with the lifelong management of a large number of thalassemic patients, there may be a tendency for pressures to be put on populations to evolve programs of this type. It is vital that this type of coercion be avoided.

Thus, while many problems remain, it should be possible, by the establishment of North-South partnerships and local networks, to evolve programs for the better control and care of thalassemia in the future (WHO 2002).

The Contribution of the Thalassemia Field to an Understanding of Genetic and Acquired Disorders Unrelated to Hemoglobin

As well as providing the first hint as to the likely repertoire of mutations that underlie monogenic disease and proof of principle that disease loci may be traced by their linkage to restriction fragment-length polymorphisms (Kan and Dozy 1978), the hemoglobin field has contributed toward an understanding of a number of nonhemoglobin disorders.

α Thalassemia and Mental Retardation

When we first encountered three North European families with Hb H disease—a moderately severe form of α thalassemia—and mental retardation, it was not clear whether this was a chance association or whether the two conditions shared a common etiology (Weatherall et al. 1981). Because of the unusual pattern of inheritance and because α thalassemia is rare in individuals of European origin, it was suggested that the two conditions might be connected and that an understanding of the molecular basis of this unusual form of α thalassemia might shed some light on the reasons for the associated mental retardation. After the first publication stimulated others to search for this association, it gradually became apparent that there are two specific syndromes associated with α thalassemia and mental retardation, which are encoded on chromosome 16 (ATR-16) and on the X chromosome (ATR-X), respectively (Wilkie et al. 1990*a*, 1990*b*; Higgs, 2001).

ATR-16, which is usually associated with mild-to-moderate mental retardation without any particular pattern of associated developmental abnormalities, is caused by subtelomeric deletions or chromosomal rearrangements of the short arm of chromosome 16 that involve the α globin–gene cluster and a number of other defined loci. These findings suggested that submicroscopic, subtelomeric deletions might be the basis for a significant number of hitherto-unexplained cases of mental retardation; subsequent studies confirmed that this is the case (Flint et al. 1995).

ATR-X is characterized by relatively severe mental retardation, characteristic facial deformities, skeletal abnormalities, and, in some cases, abnormal genitalia. The gene that is involved, *ATRX,* located at Xq13.3, encodes a member of the SW12/SNF2 family of helicases (Gibbons et al. 1995, 1997). Like other proteins of this type, ATRX has—in addition to a PHD region—a DNA-binding region and an ATPase/helicase domain, reflecting its ATP-dependent nucleosome-remodeling and DNA-translocase activities. The ATRX protein is associated with the nuclear matrix during interphase (McDowell et al. 1999) and with condensed chromatin and shows interactions with HP1 and EZH2, heterochromatin-binding proteins. Its probable role is related to accessibility of various DNA methyltransferases to chromatin; patients with ATRX show defective methylation of rDNA and other repetitive sequences (Gibbons et al. 2000). They have a very mild form of α thalassemia that is sometimes characterized by a small proportion of cells with Hb H inclusions; similar findings, though even milder, can often be identified in female carriers.

ATR-X, which has been shown to be involved in a number of other X-linked syndromes associated with mental retardation (Ausió et al. 2003), is therefore the first of a growing list of syndromes characterized by disordered chromatin remodeling.

α Thalassemia and Myelodysplasia

The association of a form of α thalassemia with a myelodysplastic syndrome in elderly patients has been recognized for many years. Early work suggested that there might be a complete absence of α-chain production in the affected cell lines in this disorder (Weatherall et al. 1978). Until recently, it was difficult to isolate the appropriate cell populations to examine the molecular pathology of this condition. However, recent work utilizing microarray analyses has shown that this condition results from somatic mutations of *ATRX* (Gibbons et al. 2003). These studies provide clear evidence that null mutations of *ATRX* are associated with a profound defect in α-globin production, indicating that the ATRX protein plays an essential role in α chain synthesis. These findings are compatible with the observation that *Atrx* knockout embryos in mice that express no full-length proteins do not develop beyond 9 days. It appears, there-

fore, that constitutional null mutations of *ATRX* are lethal, while somatic mutations that involve the hematopoietic cell line may be associated with neoplastic transformation of the bone-marrow progenitors. The mechanisms for the latter changes, and whether other genes are involved, remain to be determined.

β Thalassemia and Trichothiodystrophy

Recently, it has been found that the mutations in *XPD* that cause trichothiodystrophy (TTD) are frequently associated with a reduced expression of the β-globin genes and with the clinical phenotype of heterozygosity for β thalassemia (Viprakasit et al. 2001). The XPD protein is a subunit of the basal transcription factor, TFIIH. As well as the phenotype of β thalassemia trait, these patients have reduced levels of β-globin synthesis and β-globin mRNA. The transcription factor TFIIH is involved in both basal transcription and DNA repair. These observations provide the first evidence for reduced expression of a specific gene in TTD and support the concept that many of the clinical features of this condition result from inadequate expression of a diverse set of highly suppressed genes.

Lessons for the Future

By analyzing the molecular basis of unusual thalassemia phenotypes, considerable light has been shed on the pathogenesis of some forms of mental retardation associated with dysmorphological features and on other complex syndromes. These findings illustrate the importance of the detailed exploration of cases in which unusual phenotypes appear to be associated with common genetic disorders. It is quite possible that other developmental disorders result from mutations in genes for ubiquitous regulatory proteins, some of which may also be involved with globin-chain synthesis. Detailed morphological studies of the blood are, therefore, well worthwhile pursuing in any patient with unexplained syndromes of this type; ATRX was missed for many years simply because there seemed to be no reason for carrying out careful hematological analysis in patients with mental retardation.

Conclusions

A great deal is known about the molecular pathology of the thalassemias and about the genetic factors that modify their phenotypes. Like for most monogenic diseases, the role of the environment in phenotypic variability has been neglected, although there is increasing evidence that this may be of major importance. The central problem now is how to transport the practical applications of all this information for the benefit of patients in countries with particularly high frequencies of these conditions, notably those of Asia.

The definition of the different syndromes of α and β thalassemia associated with genetic diseases that do not, at first sight, appear to be associated with any hematological abnormalities is also of considerable importance, offering, as it does, the possibility of obtaining insights into the etiology of these conditions. Hence, the thalassemia field, as well as its great importance to many populations in the developing countries, will continue to provide valuable information for clinical genetics in general.

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