Thalassemia: The Consequences of Unbalanced Hemoglobin Synthesis*

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THALASSEMIA major or Cooley's anemia is a severe, usually fatal form of inherited anemia. Beginning approximately two to four months after birth, the disease is associated with profound anemia, jaundice, splenomegaly, expanded marrow space, siderosis and cardiomegaly. The peripheral smear is characterized by extreme poikilocytosis, anisocytosis and anisochromia. Target cells and nucleated erythroid precursors abound. Transfusion therapy is mandatory in most cases. Splenectomy may be necessary if marked splenomegaly causes destruction of platelets, white cells or transfused red cells, but the basic anemic process is usually unchanged by the procedure.

The devastating clinical effects and the fascinating genetic and pathophysiologic aspects of this severe disease and the various "thalassemia syndromes" [*1*] have led to multiple probes of these disorders by geneticists, molecular biologists and physicians. The following discussion of thalassemia has as its chief purpose a re-emphasis of the morphologic, erythrometabolic and erythrokinetic abnormalities which attend the disease. Severe thalassemia is less a disorder of *depressed* hemoglobin synthesis and more a disorder of *unbalanced* hemoglobin synthesis. Indeed, it is our view that the untoward sequellae of thalassemia are less due to underproduction of normal hemoglobin than to overproduction of aberrant hemoglobin.

To support these remarks it is necessary to present a brief review of the genetic basis of the common thalassemia disorders.

GENETICS OF HEMOGLOBIN SYNTHESIS AND THALASSEMIA

The major component of a normal hemolysate (hemoglobin A) is comprised of two pairs of homologous subunits, alpha and beta chains. The amino acid composition of these subunits has been established. To each subunit a heme ring is attached. Three other hemoglobins are present in smaller amounts in normal hemolysates. Hemoglobin F comprises less than 1 per cent of normal adult hemolysates and is composed of two alpha and two gamma subunits. Hemoglobin A_2 comprises less than 3 per cent of the hemoglobin of a normal hemolysate and is formed by two alpha and two delta chains. Hemoglobin A_3 (approximately 10 per cent of the total hemoglobin) has the same subunit composition as hemoglobin A and is probably a slightly denatured derivative of hemoglobin A. In addition, glutathione is bound in disulfide linkage probably at the site of the thiol groups of the A_3 beta chain. A_3 is found in aged red cells and is considered part of the A fraction in any clinical or genetic analysis of hemoglobin heterogeneity. (For detailed reviews, see [*1–3*].)

In the past decade it has been recognized that there are four homologous pairs of loci concerned with the production of the subunits of globin in normal human erythroblasts. These loci, termed alpha, beta, delta and gamma sites may be jointly or severally affected by a thalassemia lesion which leads to reduction of the net output of a subunit polypeptide. For simplicity we refer to the term beta thalassemia to define suppression of beta subunit production. Thalassemia major or Cooley's anemia, for example, is now thought to be due to heritable reduction of the output of both loci governing the synthesis of the beta chains of hemoglobin A. Thalassemia minor, or Cooley's trait, is due to reduction of the output of only one beta locus. These concepts have been widely accepted and provide a working basis for the explanation of many of the phenomena observed in thalassemia.

The exact nature of the suppression of the

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net output of a locus is unknown. Several possibilities exist. There may be loss of the section of the chromosome which bears all or part of the locus which is responsible for the encodement of the messenger RNA for one of the subunit polypeptides. The loss may be due to deletion or to crossing over. In support of the latter mechanism are the findings regarding the Lepore trait [*4,5*] and the hemoglobin Pylos syndrome [*6*] which are forms of beta thalassemia trait (one beta locus involved) with an accompanying abnormal hemoglobin. The abnormal hemoglobin is comprised of a normal alpha but a distinctly abnormal "beta" chain. The beta chain has been found to be an amalgam of pieces of beta and delta chains. A reasonable cause of this phenomenon would be crossing over with insertion of a piece of delta site into the midst of a beta site. On the other hand, were crossing over to be the most common cause of loss of beta chain production, more cases of the nature of Lepore trait and Pylos syndrome might be expected. In the past few years the Jacob-Monod model [*7*] has been invoked to provide a more satisfactory explanation for depressed beta subunit accumulation. On the basis of this model it is proposed that an altered repressor of the operator which controls the output of the structural beta subunit locus provides the basis of the disorder. Proof of such a model in human erythroblasts has not as yet been obtained. Another theory explores the possibility that the relatively stable messenger RNA [*8*] of human reticulocytes might become unstable. A marked instability of beta chain messenger due to the inherited production of an as yet undetermined substance could be responsible for deficient beta chain production. Itano [*2*] and Ingram and his associates [*9*] have suggested that thalassemia may be due to a single base substitution within a given triplet of the beta locus. Epstein [*10*] has recently examined the concept of a single base substitution in mutations, a review to which the reader is referred. Such a substitution would produce a template or messenger RNA which in turn would encode for the same amino acid found in the normal beta chain but be necessarily attended by a transfer RNA which might be produced in much smaller quantity than is the transfer RNA necessary for the usual triplet sequence. This new template RNA would occupy space on the ribosomes and block the ribosomal adhesion of normal RNA. This theory is extremely attractive since it would permit many different sites of delay in the assembly of beta chains and would therefore partially account for the variable severity of Cooley's anemia. It is of interest that normal beta chain production is associated with a delay point near the ninetieth amino acid residue whereas no delay points are associated with alpha subunit assembly [*11*]. On the other hand, the theory does not by itself account very well for the rise in fetal and A_2 hemoglobin observed in Cooley's anemia. Another possible genetic lesion which might occur in thalassemia is specific ribosomal injury. Such an injury might also induce a single base change in messenger RNA of the type envisaged by Ingram and his associates. Streptomycin has been shown to injure cell ribosomes in a fashion which alters single base sequence enough to lead to a "mis-sense" code and amino acid substitution within polypeptides [*12*]. The studies of Bank and Marks [*13*] provide no support for such ribosomal damage in thalassemia.

A fundamental and fortunate accompaniment of the decreased beta chain production in Cooley's anemia is an associated rise in fetal hemoglobin. Several theories have been proposed for the persistence of gamma chain production in Cooley's anemia, but no satisfactory reason for this fortunate circumstance has been established. The concentration of fetal hemoglobin may range from 10 to over 90 per cent of the total hemoglobin present in the peripheral blood. It is important to realize that the cellular distribution of this fetal hemoglobin in Cooley's anemia is different from that observed in the relatively harmless anomaly, "hereditary persistence of fetal hemoglobin." In the former the fetal hemoglobin is distributed heterogeneously, whereas in the latter each cell has very nearly the same content of fetal hemoglobin.

CLINICAL VARIETIES OF BETA THALASSEMIA

Full expression of clinical severity of thalassemia (thalassemia major) requires that the patient inherit an abnormal allele from each parent. In thalassemia minor only one abnormal allele is inherited and the disease much less severe. The heterozygous or trait forms of thalassemia are usually accompanied by moderate hypochromia and anisocytosis. In the trait form of "classic" (A_2) thalassemia a distinct rise in A_2 and a small increase in F hemoglobin is usually observed [*14*]. In F thalassemia trait, hemoglobin F is increased and hemoglobin

A_2 is normal. In a few cases both are distinctly elevated. Splenomegaly is either mild or absent, and the disease is well tolerated. The red cell life span is normal or nearly normal [*15*]. Jaundice is rare and when present [*16*] may mainly be due to ineffective erythropoiesis. The blood smear resembles that of iron deficiency except that a greater degree of poikilocytosis usually exists at a given hemoglobin level. Since thalassemia trait leads to suppression of one of the two sites of beta subunit production, doubly heterozygous subjects with combined thalassemia minor and a beta-chain hemoglobinopathy trait present a predictable picture. The production of normal beta subunits is markedly curtailed by two different abnormalities affecting the two beta production sites. Therefore, little normal hemoglobin is produced. Accordingly, a higher proportion of the hemoglobin that *is* produced is of the beta hemoglobinopathic variety.

Most cases of thalassemia major and thalassemia minor may be easily distinguished from each other but there is a small group of patients with disease of intermediate severity. For example, the union of two patients with thalassemia minor, one with increased A_2 production and the other with increased F production, may produce normal children, children with either A_2 or F thalassemia trait or children with moderate anemia, jaundice and splenomegaly and over 90 per cent fetal hemoglobin [*17*]. The latter group of patients seem to be double heterozygotes for two different beta thalassemic lesions. These patients often live moderately comfortably into adult life. In some cases parents with thalassemia minor of the high A_2 variety may produce children with all the stigmata of thalassemia major including jaundice, splenomegaly and moderately severe anemia. But for some unknown reason these children seem to weather the storm of growth and adolescence and, although they emerge with clear-cut thalassemia major, they have stable hemoglobin values as high as 9 to 11 gm. per cent and may have highly productive lives. Therefore, it behooves the physician to be optimistic in his care, with the hope that a given affected child will be one of these so-called "thalassemia intermedias." Just as the homozygous forms are sometimes subclassified as thalassemia major and intermedia, the heterozygous forms may be subclassified as thalassemia minor and minima. The terms are useful and self-explanatory.

DELTA AND GAMMA THALASSEMIA

Although the terms Cooley's anemia and Cooley's trait have been applied principally to the beta thalassemia syndromes it may be considered that the existence of alpha and delta subunits of hemoglobin represents a physiologic form of thalassemia in man since the product of the delta subunit gene is formed at only one-thirtieth the rate of the beta chain. On rare occasions complete absence of delta subunit production may be noted [*18*]. Obviously there are no clinical sequellae of this abnormality. The gradual suppression of the output of gamma chains observed during the maturation of the normal infant might be considered a form of gamma thalassemia.

ALPHA THALASSEMIA

Three important clinical syndromes result from depression of production of alpha chains. Alpha thalassemia trait, presumed depression of the net output of one of two alpha subunit sites, is similar in certain respects to beta thalassemia trait. Mild microcytosis, erythrocytosis, poikilocytosis and hypochromia are present, with microcytosis usually being the most prominent finding. There is no enhanced production at the gamma or the delta site. Consequently, no increase in A_2 or F hemoglobin is detected in adult hemolysates. In fact the total amount of A_2 hemoglobin may be reduced. The most important disorder to be differentiated from alpha thalassemia trait is that of iron deficiency. The latter is ruled out most definitively by examination of appropriate stains of marrow. Italians, Greeks, Chinese and Negroes are the most commonly affected groups. When combined with a beta hemoglobinopathy such as sickle hemoglobin, alpha thalassemia trait does not lead to an increased percentage of the abnormal hemoglobin in the hemolysates. In fact, the percentage of abnormal hemoglobin may be somewhat lower than that usually observed. In the circumstance in which it is combined with a hemoglobinopathy affecting the trans-alpha site (hemoglobin I) an increased percentage of the abnormal hemoglobin does occur.

When matings of two afflicted subjects occur, the result of such a union may be a patient with homozygous alpha thalassemia. In this fatal disease alpha subunit production is either markedly curtailed or totally absent. Gamma chain production does occur and the resulting soluble hemoglobin is Bart's hemoglobin, a tetramer of

gamma chains. Bart's hemoglobin has virtually no Bohr effect and has the additional unhappy ability to bind oxygen nearly irreversibly at the oxygen tensions of the tissues [*19*], hence conducts the function of oxygen transport extremely poorly. The result is that such infants are usually born dead or dying at approximately the thirty-second week of pregnancy bearing all the evidence of severe hydrops fetalis whether anemia is severe or moderate. Extreme macrocytosis, anisocytosis and anisochromia, with marked erythroblastemia, are present. The liver is huge, the spleen remarkably small. It should be pointed out that Bart's hemoglobin may be quite easily detected in the hemolysates of infants with the alpha thalassemia trait, but it disappears when normal gamma chain production virtually ceases and is not routinely detectable in the hemolysates of adults with pure alpha thalassemia trait. When highly sensitive technics are used, Bart's hemoglobin may be detected in a large proportion of the hemolysates of premature and newborn infants who have no familial evidence of alpha thalassemia [*20*]. This implies that gamma chain production normally outstrips alpha chain production during fetal life, and that the small gamma chain excess forms Bart's hemoglobin.

Another form of alpha thalassemia syndrome is hemoglobin H disease. This disorder also occurs in the family setting of alpha thalassemia trait, but probably cannot be regarded either as a severe form of alpha thalassemia trait or a mild form of homozygous alpha thalassemia. The disease resembles thalassemia intermedia. All the signs of hemolysis are present together with evidence of a disorder of hemoglobin production. When hypochromic hemoglobin H blood samples are exposed to a redox agent such as brilliant cresyl blue, characteristic robin's egg blue fine precipitates appear within nearly all the cells. The larger cells appear to have more precipitates. Starch block electrophoresis of hemolysates at pH 7 reveal that the hemolysates contain an abnormally rapid fraction which may include from 5 to 25 per cent of the total and which has been shown to be comprised of beta subunit tetramers. This so-called hemoglobin H has several unusual properties. Like hemoglobin Bart's, it is a useless respiratory pigment. Its lack of Bohr effect and its high affinity for oxygen inhibits its capacity to deliver oxygen to the tissues at physiologic pH or oxygen tension [*21*]. Therefore, as noted by Gabuzda [*22*], hemoglobin H traverses the circulation "forever in unhappy union" with oxygen. Hemoglobin H is an unstable tetramer and is readily oxidized within the red cell. It therefore precipitates during the life span of the cell. As it precipitates, it apparently binds glutathione to its thiol groups in a final embrace du mort [*22*]. The results of the intracellular precipitation of such a substantial quantity of hemoglobin are large intracellular Heinz bodies which may be most easily observed in the peripheral blood of splenectomized patients with hemoglobin H disease. The relationship of Heinz body formation to hemolytic anemia and hemoglobin H disease will be discussed subsequently.

The mode of inheritance of hemoglobin H disease is not at all clear. One of the parents of the patient usually has alpha thalassemia trait and the other appears normal. The apparently normal parent seems to carry a defect which permits the expression of hemoglobin H in a child who otherwise might have only alpha thalassemia trait. The defect may be one which depresses the output of the trans-alpha site, or increases the output of the unaffected beta sites. Hemoglobin H disease, then, is a form of alpha thalassemia in which the imbalance between alpha and beta subunit production is greater than that observed in ordinary alpha thalassemia trait. Hemoglobin H may be detected after exposure to brilliant cresyl blue in a small percentage of the erythrocytes of patients with ordinary alpha thalassemia trait. In fact, the detection of these cells constitutes a major clue in establishment of the diagnosis of alpha thalassemia trait. Presumably these rare cells are the progeny of erythroblasts which were so seriously affected by a gross imbalance of beta and alpha production that the excessive beta chains form the tetramer hemoglobin H.

The forms of alpha thalassemia serve as a convenient and constructive example of a concept of the pathophysiology of thalassemia which this review intends to illustrate. The basis of this concept is the central role of unbalanced subunit production in the pathogenesis of these disorders; unbalanced production in which the subunit synthesized in excess is as important if not more important in the pathogenesis of disease than is the underproduced subunit. To refine this illustration we must return to a morphologic and erythrokinetic evaluation of Cooley's anemia.

MORPHOLOGY AND ERYTHROKINETICS IN COOLEY'S ANEMIA

The appearance of the peripheral smear in Cooley's anemia depends upon the presence or absence of the spleen. When the spleen is intact, there are numerous tear-drop forms. (Fig. 1 and 2.) The red cells are heterogeneous with respect to hemoglobin content and size. Large pale cells and small dense cells may be observed together with intermediate forms. Nucleated red cells are usually present in small numbers. In wet preparations viewed with Nomarski optics, the tear-drop cells may be very striking, and contrast sharply with the cells observed following splenectomy. (Fig. 2.) The acid elution technics of Betke and Kleihauer [*23*], which define the cellular content of fetal hemoglobin, are somewhat confusing in cases of Cooley's anemia. Just as the peripheral smear stained with Romanowsky dyes shows marked heterogeneity of hemoglobin distribution so does the Betke preparation. (Fig. 3.) As a result, it may be surmised that some cells contain more fetal hemoglobin than others. Incubation of the Cooley's anemia erythrocytes of patients with intact spleens in 1 per cent methyl violet at 37°C for 10 minutes reveals few cells containing Heinz bodies.

Measurements of the autologous life span of Cooley's anemia erythrocytes in patients whose spleens are intact usually reveal biphasic curves. Cr^{51} survival studies may be more impressive than C^{14}-glycine technics in revealing the fact

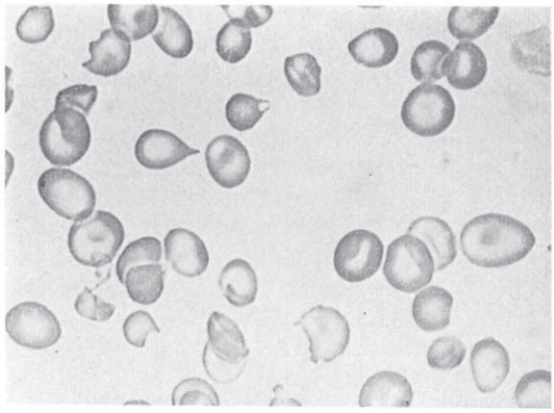

FIG. 1. Peripheral smear of a patient with Cooley's anemia and an intact spleen. Tear-drop forms are evident as well as anisochromia and target cells. Note the tear-drop form with a vacuole at its tip. (See text.)

that there is a population of cells which is rapidly removed from the circulation and another with a much longer life span [*24*]. The glycine-2-C^{14} studies do reveal, however, that the survival of hemoglobin A is considerably shorter than that of hemoglobin F [*25*]. Therefore, the heterogeneous distribution of hemoglobin F in thalassemic cells has a functional significance, and the cells rich in hemoglobin enjoy a more favorable survival.

In splenectomized patients the morphology of the peripheral blood in Cooley's anemia is markedly different from that observed in non-splenectomized patients. Tear-drop formation is not as impressive. Instead, there is an increase

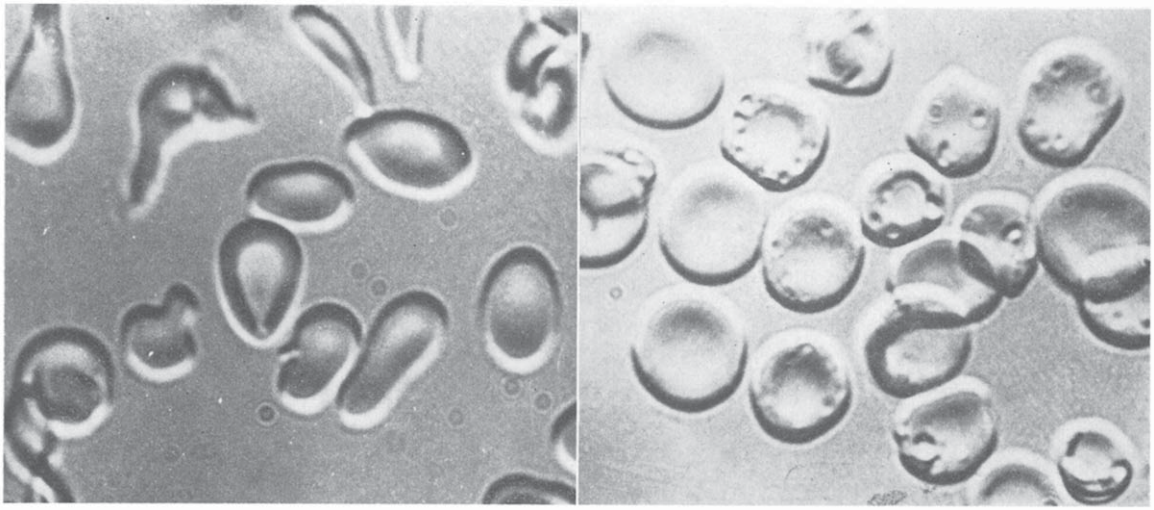

FIG. 2. Nomarski optics view of the cells of two patients with Cooley's anemia; one with an intact spleen (*left*) and the other splenectomized (*right*). Note the tear-drop formation in the patient with an intact spleen and the crateral distortions in the splenectomized patient.

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