The Diagnosis of Thalassemia Trait by Starch Block Electrophoresis of the Hemoglobin

By PARK S. GERALD and LOUIS K. DIAMOND

THALASSEMAIA trait (thalassemia minor, microcythemia) is a hereditary anemia associated with abnormally shaped erythrocytes characteristically of a reduced average cell volume.¹ It is the consensus of opinion that this syndrome is the heterozygous state for the genetic defect which in the homozygous individual results in the severe anemia known as thalassemia major.¹ Despite the generality of this belief, it is recognized that anemias otherwise indistinguishable from thalassemia have been encountered with clinical course intermediate in severity between the extremes of the major and minor disease, and hence of doubtful classification as to genetic status.² This divergence from the simple clinical dichotomy predicted by genetic theory has led to the postulation of genetic and environmental modifying factors and to speculation concerning the existence of variants of the thalassemia gene.² Such theorizing, however, has been as fruitless as it has been frequent, largely due to the lack of a satisfactory criterion for the diagnosis of “thalassemia trait.” In the absence of a suitable diagnostic test, a homogeneous group of case material has not been definable, nor the significance and cause of the clinical variation assessable.

Recently, a major advance in our knowledge of thalassemia was made by Kunkel and Wallenius who observed a characteristic alteration in the hemoglobin electrophoretic pattern in thalassemia heterozygotes.³ With their technic of hemoglobin electrophoresis in a layer of starch prepared from a settled suspension, they were able to show that a small fraction of the hemoglobin in all normal individuals can be separated from the major portion. In thalassemia trait patients the amount of this minor fraction was noted to be significantly increased above the normal value.

The present report details the results obtained with this technic, in addition to conventional hematologic measurements, in a study of a group of adults with thalassemia trait who are also the parents of children with classical thalassemia major. An increase in the minor hemoglobin fraction and a reduced mean cell (erythrocyte) volume were found to be constantly present in this patient group, and are suggested as being suitable minimum diagnostic criteria for thalassemia trait.

METHODS

The patients studied were the parents of children with thalassemia major whose records were in the Hematology Clinic of the Children’s Medical Center. The diagnosis of thalas-
IAGNOSIS OF THALASSEMIA TRAIT

Thalassemia major was based upon evidence of a severe, chronic, hemolytic anemia having onset in childhood (but after birth), and associated with abnormal erythrocytes of a characteristic appearance, the presence of nucleated erythrocytes in the peripheral circulation, hepatosplenomegaly, and markedly elevated levels of alkali resistant hemoglobin. The possibility of an abnormal hemoglobin syndrome in these children was eliminated in most cases by electrophoresis of the hemoglobin. The frequency of transfusion in several cases rendered study of endogenous hemoglobin inapplicable. In all such instances the hemoglobin of both parents was examined and the absence of an abnormal hemoglobin trait in them was verified.

Total hemoglobin was determined photometrically as oxyhemoglobin, erythrocyte counts were done with conventional technics, and the hematocrit was estimated using either a macro or a microhematocrit method. Our normal values for red cell indices correspond to those given by Wintrobe (82-92 cu. microns). Alkali resistant hemoglobin was measured by the method of Singer et al.; values above 2.0 per cent are considered abnormal. In many instances the routine hematologic examinations had been performed at the time of the initial diagnosis of thalassemia in the family, and the alkali denaturation and electrophoretic studies were done on more recently obtained specimens.

Electrophoretic fractionation of the hemoglobin was performed using a modification of the technic developed by Kunink and Wallenius. Subsequent to their publication, cyanmethemoglobin was found to be quite stable under these conditions and has been used routinely by us in the present investigation. Their original methodology was modified by omitting the covering over the starch block; the block was instead enclosed in a humidity chamber. The evaporation occurring under these conditions produced an evaporative flow of buffer, counter to the direction of electrophoretic migration. The resulting countercurrent action facilitated separation of the hemoglobin components. The electrophoretic patterns associated with several common hemoglobinopathies as obtained with this technic are illustrated in figure 1. Although not illustrated, alkali-resistant hemoglobin in amounts greater than 5 to 10 per cent is visible in the starch electrophoretic pattern as a trail behind the major component. The normal adult hemoglobin pattern (at pH 8.6) shows three components: (a) the major component (also known as A); (b) the fast minor component migrating to the anode just in advance of A; and producing the characteristic "pointed" shape of the zone electrophoretic pattern; and (c) the slow minor component with mobility comparable to hemoglobin E. This E-like hemoglobin, designated the A2 component, is the portion characteristically increased in thalassemia trait. After completion of electrophoresis, each hemoglobin pattern was divided midway between the A1 and A2 components and the separated portions eluted individually by washing on a sintered glass funnel. The concentration of hemoglobin in the eluate was then estimated spectrophotometrically and the A2 fraction calculated as per cent of the total hemoglobin. All analyses were done in duplicate. The difference between paired analyses, expressed as per cent of their average value, was independent of the A2 level. The average discrepancy was found to be 8.7 per cent (based on 50 pairs), and by calculation, the standard deviation of the mean of paired analyses was estimated as 3.8 per cent of the mean value. The normal range was established with a group of 20 healthy adults (laboratory personnel) who were studied during the course of this investigation. Their A2 fractions (the result of single analyses only) ranged from 1.7 per cent to 3.1 per cent, with an average of 2.4 per cent.

RESULTS

The results of the electrophoretic and hematologic analyses in 23 unrelated adults, all parents of children with thalassemia major, are given in table 1. Although the majority are descendants of Italian immigrants, a few claimed ancestral origin from other countries, including Central Europe, France, and Greece.

One of the group (a putative father) had a normal hematologic picture and an A2 level in the normal range. Subsequent blood grouping tests gave conclusive
proof that this man was not the genetic father of the child with thalassemia (exclusion was based upon homozygosity for the c factor in the putative father—and absence of this factor in the child as well as development of anti-c following repeated transfusions). (See case 4, table 1.)

The A2 levels in table 1 range from 3.3 per cent to an extreme of 6.8 per cent. If this range is due to a random distribution, then considerable overlap with normals can be expected, and the diagnostic value of the test is lessened. Close inspection of the distribution of the A2 value indicates, however, that a discontinuous rather than a random distribution is present since there is a definite gap
Table 1.—Hematologic Findings in the Parents of Children with Thalassemia Major

<table>
<thead>
<tr>
<th>Propositus</th>
<th>Parent</th>
<th>Hgb (Gm./100 ml.)</th>
<th>MCV (cu. microns)</th>
<th>MCHC (%)</th>
<th>Retics (%)</th>
<th>Alkali-resistant Hgb (%)</th>
<th>A2 Content (% total Hgb)</th>
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<tr>
<td>Case 1</td>
<td>Father</td>
<td>13.6</td>
<td>58</td>
<td>32</td>
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<td></td>
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<td>3.2</td>
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<td>56</td>
<td>30</td>
<td>1.8</td>
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<tr>
<td></td>
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<td>80</td>
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<td>2.1</td>
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<td>5.0</td>
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<tr>
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<td>9.4</td>
<td>68</td>
<td>31</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Case 4</td>
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<td>*</td>
<td>*</td>
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<td>2.4</td>
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<td>67</td>
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* See text (Results).

Frequency Distribution

Fig. 2.—Distribution of values for Hgb A2 content in parents of children with thalassemia major. The points correspond to the data given in table 1.

from 3.8 per cent to 4.4 per cent (fig. 2). The significance of this gap was further attested when serial analyses in four individuals, representing a wide range of A2 content, showed essentially no change over periods varying from 3 to 18 weeks. Such discontinuity suggested the existence of genetic factors, and, accordingly,
family studies to assess the intrafamilial correlation of the A2 fraction in thalassemia trait members were begun. To date, six thalassemia trait pedigrees have been examined, including a total of 14 affected members. The A2 value in the affected members was very similar within individual pedigrees (fig. 3). The wide distribution of A2 values in table 1 thus appears to be the consequence of including genetically different groups of thalassemia trait. Within each group, the hemoglobin fractionation results fall within a narrow range. This justifies drawing a sharp distinction between normal individuals and the group of thalassemia traits with slightly elevated A2 values.

DISCUSSION

An attempt to determine minimum diagnostic criteria for “thalassemia trait” implies prior knowledge of the definition of “thalassemia trait”—an obvious contradiction. As a practical solution to the problem, we chose to study the most homogeneous form of a thalassemia syndrome with which we were acquainted—namely, thalassemia major. If one assumes that thalassemia major is the result of homozygosity for the thalassemia gene, then the parents of such children should all be affected with thalassemia trait. As represented by the group of parents included in table 1, the heterozygous state for the thalassemia
gene is invariably associated with a reduced mean cell volume and an increase in the A2 fraction. Homogeneity with respect to both of these criteria lends credence to our belief that these cases are similarly homogeneous in etiology.

Although the data given above are an adequate basis for initiating the hypothesis that an elevated A2 level is a necessary criterion, they are inadequate for the evaluation of the sufficiency, i.e. the pathognomonic value, of the test. Concurrently with the present study, several hundred blood specimens from pediatric and adult patients with assorted hematologic and other diseases were examined. In every instance of increased A2 content for which hematologic data were available, the presumptive diagnosis of thalassemia trait could be made. Josephson, however, has reported finding an increased A2 value in pernicious anemia in relapse. Any possible confusion with this disease would, of course, be prevented by determination of the mean cell volume.

The rather small difference in A2 values between some thalassemia trait patients and some normals is of considerable interest. The clinical consequences of the presence of the thalassemia gene can scarcely be the direct result of the altered proportion of the hemoglobin components, unless a threshold value exists for the effect of this alteration. Clinically, we have not been able to classify the heterozygotes listed in table 1, nor their progeny, in any way to correspond with the grouping according to A2 level. If such a threshold exists, it would have to be very narrow indeed for the “low-valued” heterozygotes to exert their full clinical effect. The altered hemoglobin fractionation pattern observed in pernicious anemia in relapse likewise argues against a close relation between clinical symptomatology and the disturbed hemoglobin synthesis. From this we conclude that the effect of thalassemia upon the synthesis of normal hemoglobin components is a secondary one.

Although microcytosis is generally considered to be a manifestation of thalassemia trait, absence of microcytosis has been noted occasionally in pedigrees of anemias clinically resembling thalassemia. With the discovery of the combined abnormal hemoglobin syndromes, one explanation for these exceptions became available. This explanation, however, has not sufficed to account for all exceptions. In a number of published pedigrees, thalassemia has been diagnosed in the absence of microcytosis when other properties commonly attributed to the thalassemia gene, such as increased osmotic resistance, increased number of target cells, or interaction with abnormal hemoglobin trait, have been present. Admittedly, nonpenetrance of microcytosis due to thalassemia has apparently been observed and has been stressed as a possible explanation for such anomalous pedigrees. Nonpenetration does not seem reasonable, however, when none of the so-called thalassemia trait members of a family exhibit microcytosis. We ourselves have not been able to find any instances of nonpenetrance, despite a rather intensive search. Consequently, we feel that many of the normocytic abnormalities labelled “thalassemia trait” are unknown hereditary erythrocytic defects awaiting identification.

The very recent article by Singer et al., in which a patient with “thalassemia minima” but without microcytosis is described, is most provocative. In this paper, they report on a Negro family in which the father has Hgb C+S disease and the mother has “thalassemia minima.” Among their progeny are one case
each of “Hgb S-thalassemia” and “Hgb C-thalassemia” disease. The diagnosis of thalassemia minima in the mother was established by the finding of “some ovalocytosis and the presence of numerous target cells (26 per cent)” and by the elevation in A₂ content (9.2 per cent!). The absence of microcytosis in the presence of an increased A₂ is at variance with our findings. The explanation may lie in the following unusual features of this patient: (1) the high frequency of target cells (in a survey of 20 thalassemia traits, only one was found to show a comparable number of target cells; the remaining 19 had but a few per cent); (2) the very high level of A₂ (compared to a maximum of 6.8 per cent in our series); and (3) the racial origin (no Negro patients were included in our series). As has been suggested by Zuelzer et al., there is clinical evidence to indicate that “thalassemia” in Caucasians may differ from that observed in Negroes. If we are correct in concluding that the elevation in A₂ content is only secondarily related to the basic disturbance in thalassemia (as we have defined the disease), then it is quite conceivable that variants of thalassemia may be associated with an elevated A₂ value, but be otherwise distinguishable from thalassemia trait.

This emphasis on the presence of microcytosis in thalassemia trait must not be construed as indicating that a reduced mean cell volume is a sufficient criterion for the diagnosis. During the present study of thalassemia, two varieties of hereditary microcytosis electrophoretically distinguishable from thalassemia were encountered. Since they serve to illustrate the value of starch block electrophoresis, a brief account is appropriate. The first of these was discovered in the family of a patient with Hgb H disease. One parent of the patient was hematologically normal and the other resembled thalassemia trait—in conformity with published reports of this disease. The hemoglobin fractionation pattern of the microcytic relatives of the patient revealed that a normal level of the A₂ component was present, as opposed to the increased value found in thalassemia major pedigrees. On the basis of this finding, the hereditary defect has been classified as a non-thalassemic hereditary microcytosis.

A second microcytic abnormality transmitted as a dominant characteristic occurred in the family of a child with classic thalassemia major. The mother and four of her relatives possessed microcytic erythrocytes, and on electrophoresis of their blood a new abnormal hemoglobin present in low concentration (10 to 12 per cent of the total pigment) was discovered. Subsequently, the hemoglobin of the propositus was also found to contain a small amount (5 per cent) of the abnormal hemoglobin in addition to the increased alkali-resistant fraction (80 per cent). Both of these hereditary microcytoses will be described in detail in the near future.

Now that means is available for making a positive diagnosis of thalassemia trait, use of the term should be limited to those cases which fulfill the minimum diagnostic criteria given above. Variants of thalassemia syndromes, in particular, should be reinvestigated to determine if they are not actually nonthalassemic diseases, two of which have already been recognized.

SUMMARY

Starch block electrophoresis of the hemoglobin has been performed for a group of adults who are the parents of children with thalassemia major. The hemo-
globin electrophoretic pattern was found to be constantly abnormal in this group, in that the minor component with E-like mobility (designated the $A_2$ component) constituted a greater than normal proportion of the total hemoglobin. Reduced mean cell (erythrocyte) volume was likewise found to be present in all members of the patient group.

If it is assumed that typical childhood thalassemia major represents the homozygous state for the thalassemia gene, then the patient group studied constitutes a population of adults heterozygous for thalassemia. Since microcytosis and an increase in the $A_2$ content were constantly present in this group, they are suggested as suitable minimum diagnostic criteria for thalassemia trait.

The degree of elevation of the $A_2$ fraction was noted to have a discontinuous distribution. Preliminary studies have demonstrated that the degree of elevation appears to be identical in affected members of single pedigrees. The discontinuity in distribution observed is thus apparently under genetic control.

The rather small difference in $A_2$ content between some normal and some thalassemia trait adults implies that the alteration of hemoglobin synthesis is a secondary phenomenon, since such “low-valued” thalassemia traits were not different in their clinical expression. Despite the smallness of this difference, quantitation of the $A_2$ fraction sufficed to distinguish these “low-valued” thalassemia heterozygotes from normal individuals.

**SUMMARIO IN INTERLINGUA**

Electrophorese de hemoglobina per medio del technica a bloco de amylo esseva effectuate pro um gruppo de adultos qui es le parentes de patientes de thalassemia major. Esseva trovate que le configuration electrophoretic del hemoglobina esseva uniformemente anormal in iste gruppo, i.e. le componentem con mobilitate simile a E (designate como componente $A_2$) constitueva un proportion plus que normal del hemoglobina total. Un reduce volumine cellular medie (de erythrocytos) esseva etiam constatate in omne membros del gruppo.

Si nos suppose que typic thalassemia major de juveniles representa le stato homozygotic pro le gen de thalassemia, il seque que le gruppo de individuos hic studiate constitue un population de adultos heterozygotic pro thalassemia. Proque microcytosis e un augmento del contento de $A_2$ esseva uniformemente presente in iste gruppo, il es sugerite que ille duo factores representa un convenibile minimo de criterios diagnostic pro le tracto de thalassemia.

Esseva notate que le grado de elevation del fraction $A_2$ habeva un distribution discontinue. Studios preliminari hai demonstrate que illo es apparentemente identic in afficite membros del omne arbo genealogic. Assi le discontinuitate del distribution que hai esse observate pare esser geneticamente determinate.

Le satis micre differentias del contento de $A_2$ in certe adultos normal comparate con certe alteres con le tracto de thalassemia significa que le alteration del synthese de hemoglobina es un phenomeno secundari, proque tal casos de tracto de thalassemia a “valor basse” non differeva in lor expression clinic.

**REFERENCES**

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The Diagnosis of Thalassemia Trait by Starch Block Electrophoresis of the Hemoglobin

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