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The Distribution of Hemoglobin Types in Thalassemic Erythrocytes *

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In homozygous β-thalassemia the erythrocyte population is largely nonhomogeneous; no other condition presents such a variation of size, shape, and content of hemoglobin in the red cells. In addition, red cell morphology may differ from patient to patient as concerns degree and quality of alterations. This variability, evident at mere examination of red cell morphology, has been supported by the early studies on red cell survival, which showed that the red cell population contains very short-living as well as rather long-living cells (1). Essentially similar findings were obtained in some studies by using chromated erythrocytes (2, 3); in other studies no initial rapid fall of the chromium survival curve was observed, but there was indirect evidence compatible with the presence of a very short-lived population of red cells (4). Support for heterogeneity has also been obtained by the technique for the histochemical demonstration of hemoglobin F (5), all reports agreeing on the nonuniform distribution of fetal hemoglobin over the red cell population (6–8).

In 1963 further data concerning the heterogeneity of red cells in homozygous β-thalassemia were reported. a) It was shown that a large proportion of red cell precursors, but only occasional red cells, present Heinz-body–like precipitates of hemoglobin (9).1 Although one may consider a splenic “pitting out” mechanism for removal of the inclusions, studies on the fate of erythrocytes in hemoglobin H disease (10) and in induced Heinz-body anemias (11) failed to demonstrate such a process. Thus, the great difference observed in the percentages of inclusion carrying cells between normoblasts and erythrocytes indicates that cells with inclusion are very short-lived. b) Elaborate radioisotope studies by Gabuzda, Nathan, and Gardner (12) have shown that the various hemoglobin fractions in thalassemia display dissimilar turnover rates, the greater turnover concerning hemoglobins A and A₂. These differences in turnover rates may be due to the inhomogeneous distribution of the various hemoglobins over the red cell population. The same authors obtained evidence supporting this hypothesis by centrifugal separation of thalassemic red cells; they found that the lower red cell layer presents significantly higher percentages of hemoglobin F than the upper layer.

The present study is based on the working hypothesis that insufficient β-chain synthesis is fundamental for the causation of ineffective erythropoiesis in β-thalassemia. According to this hypothesis, which has been schematically presented elsewhere (13), the hemoglobinemic inclusion bodies represent precipitated α-chains, which have remained uncombined because of shortage of their complementary chains; since the β-chain deficiency is genetically determined, it was thought that “compensatory” formation of γ-chains would lead to increased hemoglobin in the cells, to reduction of the inclusion phenomenon, and—directly or indirectly—to a better survival of the red cells.

So far, the distribution of the various hemoglobin fractions in thalassemia in relation to the content of hemoglobin in the cells has not been studied. The principle of differential centrifugation in albumin (14) was applied to separate the red cells of thalassemia major according to their specific densities.
Methods

Patients

The study has been carried out on 14 patients with homozygous $\beta$-thalassemia, one patient with $\beta$-thalassemia/hemoglobin "Pylos" disease. Two patients with high F/$\beta$-thalassemia were also included in the study. Patients who had been transfused within the last 4 months were excluded. Three normal infants, 2 to 3 months of age, were also studied for comparison. The diagnosis was established by the clinical picture, the hematological and biochemical data, and whenever possible by family studies. Most of the patients had a characteristic mongoloid, severely anemic facies and a large—in one instance, a huge—spleen well below the left costal margin. Seven of them had been splenectomized. The age of the patients ranged from 2½ to 28 years; eight of them were males, the remaining females. Most patients had severe thalassemia, requiring transfusion at short intervals; this has been a problem for our study, since we often had to postpone a new transfusion, whenever possible, for a period of at least 4 months, so that all foreign cells could be eliminated. Three of our patients were of intermediate severity; they did not need transfusions and were almost normally active.

Procedure

Fifteen to twenty ml of blood was collected from each patient, preferably just before a transfusion, with the usual double oxalate anticoagulant. The plasma was removed by low speed centrifugation, and the red cells were suspended in 2 vol of 30% bovine albumin. Several hematocrit tubes were filled with the erythrocyte suspension. They were centrifuged at 700 rpm for 30 minutes in the cold, followed by 30 more minutes at 3,500 rpm. At the end of this procedure the erythrocytes were separated into two layers within each hematocrit tube, an upper layer (ca. ⅔ of the red cell column) containing loosely packed erythrocytes giving an impression of red dust suspended in the albumin, and a lower layer (the remaining ⅓) consisting of densely packed erythrocytes. In some instances, a layer of clear supernatant albumin was seen at the top. The upper cell layer was aspirated with a Pasteur pipette into a glass tube; then, the middle portion (about ⅔ of the total red cell column) was removed from the densely packed cells, and the remaining packed erythrocytes (the lowest ⅓ of the total column) were aspirated into another tube. The upper and lower layers were suspended again in more albumin and subjected to a second differential centrifugation from which a final "top" and "bottom" layer was obtained. The quantities of the red cells thus obtained ranged from 0.3 to 0.8 ml. The erythrocytes of these layers were washed three times with saline to remove the albumin and then with their plasma in order to regain, as much as possible, their original dimensions and shape; they were finally suspended in their plasma, and after an overnight stay at 4° C the top and bottom layers were subjected to the following measurements.

Hemoglobin was determined colorimetrically as cyanmethemoglobin.

The red blood cell count was determined by a Coulter counting apparatus under the standard conditions in this laboratory, i.e., aperture 5 and threshold 4 with an orifice of 100 $\mu$.

The volume of packed red cells was measured in capillary tubes after high speed centrifugation.

Size distribution was determined in nine cases by counting the red cells at different thresholds, increasing each step by 4 U. The differences between each count and its next one were plotted on graph paper against the threshold units; the curve thus obtained was representative of the size distribution of the cells (15, 16).

Smears from each sample were also examined after regular staining as well as after the acid-elution technique (7); in addition, smears for reticulocyte count (with cresyl blue) and thalassemic inclusion bodies (with methyl violet) were prepared.

The remaining erythrocytes of the two layers were washed repeatedly with saline and hemolyzed by addition of 2 vol of distilled water containing 0.3 to 0.5 parts of toluene. Stromata were removed by centrifugation and filtration, and the hemolysates were adjusted to identical hemoglobin concentration.

Hemoglobin F was determined according to the technique of Jonxis and Visser (17) with the only difference that hemolysate was used instead of whole blood. A Zeiss PMQ II spectrophotometer was used for all measurements.

The standard deviation of this method in our experiments has been ±1.51% for samples with low hemoglobin F levels (ca. 25%) and ±2.56% for samples with hemoglobin F about 60%. The percentage of the alkali labile hemoglobins (non-F hemoglobins) was obtained by subtraction.

From the above measurements we estimated the mean contents per erythrocyte of total hemoglobin (MCH), of hemoglobin F (MCH-F), and of hemoglobin-non-F (MCH-non-F).

Electrophoresis of all hemolysates was performed on starch gel using a discontinuous Tris-EDTA-boric acid/barbital buffer at pH 8.4 (18) and, in some instances, also on agar gel using a citrate buffer at pH 6.2 (19). In three cases, in which we obtained a greater amount of "top" and "bottom" hemolysate, hemoglobin $A_2$ was quantified after electrophoretic separation on starch block according to Kunkel, Ceppellini, Müller-Eberhard, and Wolf (20).

Results

The following differences were observed between top and bottom layers in the homozygous thalassemias and the hemoglobin E/$\beta$-thalassemia and the hemoglobin "Pylos"/$\beta$-thalassemia combinations (Table I).

Mean corpuscular hemoglobin. Erythrocytes of the top layer consistently had a lower MCH
than those of the bottom layer; there has been not a single exception. The mean MCH of the top layer was 17.73 μg, ranging from 13 to 22.3, and the mean MCH for the bottom layer was 21.75 μg, ranging from 16.8 to 25.0 μg. The difference between the two layers is statistically significant (t = 3.8, p < 0.01). There was practically no difference between the MCH of the bottom layer and the MCH of the uncentrifuged blood in all cases; this indicates that the bottom layer is more representative of the entire red cell population than the top layer, the cells of which contribute only little to the determination of MCH of the circulating erythrocytes.

**Red cell size.** We were prevented from calculating the mean corpuscular volume (MCV) and, hence, the mean corpuscular hemoglobin concentration (MCHC) of the top layers in several cases because of the inadequate packing of the red cells that had been suspended in the albumin medium, even after removal of the albumin and resuspension in their own plasma. This phenomenon was more pronounced in splenectomized patients. Therefore no comparisons between the MCV and MCHC values of the two layers were made. Since no reliable data could be obtained concerning mean cell size by measuring the value of packed red cells, we had to rely on the size distribution curves obtained by the Coulter counter. The curves obtained in nine cases indicated that the top layer contained the larger cells in five cases; an example is given in Figure 1. In the other four cases no significant differences were evident. Since the top layer presented a lower MCH than the bottom layer, it can be concluded that the MCHC of the top layer was also lower, especially when the cells of the top layer were larger.

### TABLE I

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>MCH*</th>
<th>Hemoglobin F</th>
<th>Hemoglobin-non-F</th>
<th>MCH-F</th>
<th>MCH-non-F</th>
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<td>Bottom</td>
<td>Top</td>
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<tr>
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<td>69.0</td>
</tr>
<tr>
<td>21</td>
<td>III</td>
<td>31.3</td>
<td>34.8</td>
<td>27.0</td>
<td>65.0</td>
<td>63.0</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
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<td>31.7</td>
<td></td>
<td></td>
<td>8.45</td>
</tr>
</tbody>
</table>

|     |       | Bottom |
|     |       | Top    |

* MCH = mean corpuscular hemoglobin.
† Hemoglobins A + E + A0
‡ Hemoglobins A + A0 + "Pyllos."

**These results** indicate that the MCH of the top layer is significantly lower than that of the bottom layer. The mean MCH of the top layer was 17.73 μg, ranging from 13 to 22.3, and the mean MCH for the bottom layer was 21.75 μg, ranging from 16.8 to 25.0 μg. The difference between the two layers is statistically significant (t = 3.8, p < 0.01). There was practically no difference between the MCH of the bottom layer and the MCH of the uncentrifuged blood in all cases; this indicates that the bottom layer is more representative of the entire red cell population than the top layer, the cells of which contribute only little to the determination of MCH of the circulating erythrocytes.

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Cellular morphology. The top layer contained constantly more inclusion carrying cells than the bottom layer, in accord with previous preliminary observations (9). The difference in percentage of inclusion carrying cells was striking in the splenectomized cases, the top layer containing up to 13 times more such cells than the bottom; thus, in one case, there were 410 red cells with inclusions per 1,000 red cells in the top layer in contrast to only 33 per 1,000 in the bottom.

In contrast, normoblasts accumulated in the bottom layer, which contained from three to 16 times more normoblasts per 1,000 red cells than the top layer. There were no significant differences in the proportion of inclusion carrying normoblasts in each of the two layers.

The proportion of reticulocytes at the top exceeded consistently by five to ten times that of the bottom layer.

Apart from these differences, morphology of the red cells indicated greater hypochromia in the top.

The inspection of smears treated by the acid elution technique did not provide clear-cut information concerning differences in the two layers. It was evident, however, that the hemoglobin of the inclusion bodies could not be eluted; in this regard these inclusions are similar to other intracellular hemoglobin precipitates, irrespective of their origin (8).

Hemoglobin composition. In every instance the bottom layer had a higher percentage of hemoglobin F in comparison to the top layer and, therefore, a proportionately lower percentage of the non-F hemoglobins. The increment of hemoglobin-F in the bottom layer was always considerably higher than 3 SD of the method applied. The higher content of hemoglobin F was confirmed in all instances by starch gel or agar gel electrophoresis, which clearly demonstrated a relatively larger fraction of hemoglobin F in the hemolysate from the bottom layer (Figure 2).

Since the MCH of the two layers may differ by as much as 7 μg, it is clear that the differences in percentage do not reflect adequately enough the hemoglobin constitution. Therefore, the mean absolute amounts of each hemoglobin

FIG. 1. SIZE DISTRIBUTION OF RED CELLS IN CASE 11, AS OBTAINED BY THE COULTER CELL COUNTER. The shift to the right of the curve of the top layer erythrocytes in comparison to that of the bottom layer indicates that the upper layer contains comparatively larger red cells.

FIG. 2. ELECTROPHORETIC COMPARISON OF HEMOLYSATES FROM THE TOP AND BOTTOM LAYERS. Starch-gel electrophoresis, pH 8.2, amido black stain. Anode to the right. a) Case 10; b) Case 13. The same amount of hemolysate of equal hemoglobin concentration was used for each comparison.
type per cell were calculated (MCH-F and MCH-
non-F). The mean value of MCH-F for the
whole series was 5.39 \( \mu g \) at the top and 9.76
\( \mu g \) at the bottom layer. The difference be-
tween the two layers varied from 1.60 to 8.28
\( \mu g \) and was always in the same direction. This
difference was statistically significant \( (t = 2.82, 0.01 < p < 0.02) \).

On the contrary, the MCH-non-F varied only
little in the two layers, whether the cases had
high or low percentages of hemoglobin F. The
respective mean values of MCH-non-F for
the entire series were 12.46 \( \mu g \) at the top and 11.97
\( \mu g \) at the bottom layer. This is not a significant
difference \( (t = 0.49, p > 0.70) \). Individual values
varied slightly in both directions and were of a
magnitude that in most instances could be well
accounted for by the techniques. There was only
one case (No. 10) in which on two different oc-
casions a difference exceeding 3 \( \mu g \) was obtained,
the bottom layer having the lower MCH-non-F.

The above results are shown diagrammatically
in Figure 3. Figure 4 indicates the existence of
a parallel between the increments of MCH and
MCH-F from the top to the bottom layer. The
\( r \) between the above two parameters was found
To be 0.745, corresponding to \( p < 0.01 \); apparent-
ly, the higher MCH of the bottom layers is
largely caused by the increased amount of hemog-
lobin F.

In connection with these results, the MCH-F
of 22 patients with various forms of thalassemia
major who had not received transfusions, ex-
amined routinely on various occasions, was found
to be well correlated to their MCH \( (t = 0.73, p < 0.01) \), indicating that the level of MCH in
this disease depends to a large extent on the
amount of hemoglobin F (Figure 5).

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**Figure 3** Diagrammatic comparison of absolute amounts of hemoglobins F and non-F in the top and bottom erythrocyte layers of patients with thalassemia and combinations (Cases 1 to 16). The values of MCH-non-F deviate only little from the diagonal and are equally distributed on both sides, whereas values of MCH-F display a large deviation to the right.
Hemoglobin A\textsubscript{2} follows the pattern of hemoglobin A. On inspection of the electrophoretic patterns it was evident that in all cases the top layer presented a clearly higher concentration of hemoglobin A\textsubscript{2} than the bottom layer (Figure 2). Hemoglobin A\textsubscript{2} was quantified in three cases; although the percentage in the top layer was about twice as high as in the bottom layer, the differences became much smaller (Table II) when calculated in absolute amounts (MCH-A\textsubscript{2}). However, a slightly lower concentration of hemoglobin A\textsubscript{2} was obtained in the bottom layer.

The findings in the two cases of thalassemia/persistent hemoglobin F are not exactly imposable on those of the other cases studied. In both patients, as in homozygous thalassemia,

<table>
<thead>
<tr>
<th>CASE</th>
<th>PERCENTAGE HEMOGLOBIN A\textsubscript{2}</th>
<th>MCH A\textsubscript{2}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TOP</td>
<td>BOTTOM</td>
</tr>
<tr>
<td>Case 2</td>
<td>7.5</td>
<td>5.4</td>
</tr>
<tr>
<td>Case 11</td>
<td>7.0</td>
<td>3.5</td>
</tr>
<tr>
<td>Case 13</td>
<td>9.8</td>
<td>4.6</td>
</tr>
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</table>

the bottom layer had a higher percentage of hemoglobin F; in this respect the results are in accord with previous findings obtained by the acid elution technique, which in the same cases indicated a rather inhomogeneous distribution of hemoglobin F (21). However, the results as concerns the MCH were different, since the increase of hemoglobin F at the bottom layer was not accompanied by a proportionately higher MCH. Interpretation of these findings necessitates further studies.

For the sake of explanation the red cells of...
infants in the stage of transition from fetal to adult hemoglobin synthesis were also examined in the same manner. The bottom layer contained cells with a higher MCH-F, and the top layer had clearly higher levels of MCH-non-F; the pattern was entirely different from that observed in our thalassemic patients, and there was a reciprocal relationship between the two hemoglobin fractions (Figure 6).

Discussion

The pronounced variation in size and hemoglobin content of the red cells in thalassemia major precludes a more quantitative application of the acid elution technique (22). Therefore, although very valuable information has been gained about distribution of hemoglobin F, no equally valid information can be deduced by this technique about the distribution of hemoglobin A. Additional difficulties may be caused by the fact that under certain circumstances, for instance in normoblasts (23), normal hemoglobin A cannot be eluted from the cells and inclusion bodies are acid-resistant.

The present study, while confirming by a different approach the nonuniform distribution of hemoglobin F over the red cell population in thalassemia major, permits also the conclusion that hemoglobins A and A2 are rather uniformly distributed. It follows that there is no obvious reciprocal relationship between absolute amounts of hemoglobin F and non-F per cell and that differences in the total hemoglobin content of the cells are mainly due to the presence of variable amounts of hemoglobin F. The correlation between MCH and MCH-F, established in whole blood of patients who have not received transfusions, corroborates this concept.

The absence of a strictly reciprocal relationship between hemoglobins A and F is confirmed by comparison with the findings in the 2- to 3-month-old babies, in which the shift in γ- to β-chain synthesis is not yet complete. These findings, although similar to β-thalassemia as concerns hemoglobin F, are entirely different as concerns hemoglobin A. In such bloods, the acid elution technique shows a rather bimodal distribution of the cells (7), whereas in thalassemia it rules out the presence of two distinct red cell populations, one containing only hemoglobin F and the other only hemoglobin A.

In the present study, separation of the cells was obtained according to their density. Red cell density has been shown to depend on cellular age (14, 24, 25). An a priori application of the same principle to thalassemic cells may not be correct because of their extreme cellular diversity, and the separation may not be as clear-cut as in cases with homogeneous hemoglobin distribution. The view that in thalassemia major also the top layer contains a population of shorter mean cellular age is supported by the significantly higher percentage of reticulocytes and inclusion carrying cells in this layer; the evidence that the latter cells are very short-lived has been stated in the introduction. Some error may be caused by the concentration of normoblasts at the bottom layers, especially in the splenectomized cases; actually, their presence may have led to an underestimation of hemoglobin F of the older red cells. The separation of cells according to age by centrifugation has been accepted by Rigas and Koler (10) in hemoglobinopathy H and by Gabuzda and his colleagues in thalassemia major (12); as stated in the introduction, the latter authors obtained significantly higher percentages of hemoglobin F at the bottom layers.

We believe, therefore, that the bottom layer contained the relatively more viable cells, which have a higher MCH-F and hence a higher MCH than the cells of the top layer, which present a lower content of hemoglobin F, a lower MCH, and evidence for a surplus of α-chains (26). Cells, unable to make up their deficit of β-chains by the production of sufficient γ-chains, are, therefore, removed earlier. This is in accord with the findings of Gabuzda and associates (12) obtained by studying the turnover of the various hemoglobin fractions. Their data reveal that after an initial rapid turnover of hemoglobins A and A2 (and to a smaller extent of hemoglobin F, evident especially in one splenectomized case), the slopes of the specific activities become more or less parallel, indicating a rather similar survival of the three hemoglobins. This similarity of survival is probably due to the cells containing, in addition to hemoglobins A and A2, adequate amounts of hemoglobin F. A further indication that hemoglobin F may confer better viability to the thal-
assemic cells is found in some preliminary data from Malamos, Belcher, Gytaki, and Binopoulos (27); longer Cr$t^{4}$ values were obtained in cases having higher percentages of hemoglobin F.

Although red cell viability, in accord with the foregoing, appears to be related to the MCH, we do not maintain that the mean cellular hemoglobin concentration is the only or the primary factor determining the cell survival. The latter probably depends on other factors as well, connected to the insufficient or disordered synthesis of $\beta$- and $\gamma$-chains occurring in thalassemia or to both.

"Compensation" by synthesis of $\gamma$-chains does not appear to be an automatic result of the presence of the thalassemia genes. If synthesis of $\gamma$-chains were mediated only through the action of the thalassemia genes, one would not expect the observed pronounced differences of the MCH-F of the various cell layers, since all red cell precursors are endowed with the same genes. Recently, Marks and Burka have found that the synthesis of hemoglobin F proceeds at a similar rate in cells from thalassemic and nonthalassemic subjects in vitro (28). This finding supports the opinion that the $\beta$-thalassemia gene is not associated with a specific mechanism for compensatory $\gamma$-chain synthesis. On the other hand, the absence of large variation in the levels of MCH-non-F in the two erythrocyte layers suggests that this level is determined mainly by the mutant genes. The questions of what causes $\gamma$-chains to be synthesized and why there is great variation in the levels of hemoglobin F per cell cannot be answered without resorting to speculation and therefore will not be discussed here.

In view of the variable intensity of $\gamma$-chain synthesis, we cannot rule out the possibility that in some cells its mechanism has been stimulated maximally and has prevented synthesis of $\beta$- and $\delta$-chains from reaching its upper limits. This effect may be expected especially when $\beta$- and $\delta$-chains are produced in relatively large amounts. Such a phenomenon may have occurred in Case 10, who had the highest MCH-non-F of the whole series. The finding of some $\gamma_4$ in cases of thalassemia major (29) is a further indication that in some cells $\gamma$-chain synthesis indeed overshoots the mark. The question of whether a small proportion of cells may contain only hemoglobin F and no A and A$_2$ at all cannot be answered at the present; such a population, if very small, would not be detected by the methods applied. It is clear, however, that hemoglobin F synthesis may not proceed freely even when no synthesis of $\beta$-chains can be detected. Cases of thalassemia major without hemoglobin A were not included in this study, since they would not be sufficiently informative. In these cases also the cells differ greatly in size and hemoglobin content, indicating that the absolute amount of $\gamma$-chains varies from cell to cell; so varies the MCH from case to case. The finding of inclusion bodies in these cases may be a further indication that, at least in some cells, compensation by $\gamma$-chain production is inadequate.

The differences in the levels of hemoglobin A$_2$ in the top and the bottom layers may help to explain the paradoxical behavior of this minor component, which is increased in the heterozygotes, but is often normal in the homozygotes for thalassemia. This paradox is more apparent than real. Cells containing relatively less hemoglobin F present a high percentage of hemoglobin A$_2$ along with relatively more hemoglobin A; these cells belong to the short-lived population, and their proportion may be insufficient to greatly influence hemoglobin A$_2$ levels in standard hemolysates.

The profound inhomogeneity of the red cells in homozygous thalassemia, resulting from the present study and other studies referred to, is probably even larger, since the red cell populations examined constitute only a fraction of the cells that are born. In most cases the circulating cells are chiefly those in which the effects of the two thalassemic genes have been more or less adequately compensated for through $\gamma$-chain synthesis. Accordingly, separation of the cells by their density may serve as a more informative tool for the study of the effects of the thalassemic genes on erythrocyte constitution.

**Summary**

The distribution of hemoglobin and of the various hemoglobin types was studied after differential centrifugation of the red cells in $\beta$-thalassemia homozygotes and in the association of $\beta$-thalassemia with other hemoglobin abnormalities.
Cells of the bottom layer have a significantly higher mean corpuscular hemoglobin, a significantly higher percentage of hemoglobin F, and, hence, a higher mean absolute amount of fetal hemoglobin per cell than those of the top layer. The differences in the absolute amounts of the non-fetal hemoglobin per cell between the two layers are not significant. Increments of mean corpuscular hemoglobin and mean corpuscular fetal hemoglobin from the top to the bottom layer showed good correlation. The findings suggest that the content of hemoglobin in the red cells in thalassemia depends to a large extent on the capacity of the cells to synthesize $\gamma$-chains in addition to the genetically determined level of $\beta$-chain synthesis. The "compensation" capacity differs from cell to cell and from case to case; it appears to be important to the survival of the cells in the circulation.

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References


