Microspectrophotometric Studies of Individual Erythrocytes from Thalassemia Patients. I. A Case of Double Heterozygosity for \( \beta \)-Thalassemia and Panglobinopenia?

By Gastone Matioli and Emile Zuckerkandl

The object of the present report is to describe quantitatively the distribution of different hemoglobin polypeptide chains among single erythrocytes of a thalassemic patient. Such an analysis has not been previously performed, in spite of its importance for a better understanding of the thalassemia syndrome, whose basic mechanism has proved elusive. Since circumstances do not at present allow us to extend the application of the method used to further cases, the interest of the one to be presented prompts us to report on it. A hypothesis is proposed to account for the observed facts. Cases presumed to be analogous are found in the literature and are discussed in a separate paper.1

Methods

Single erythrocytes from the peripheral blood of the patient were analyzed for the presence and the quantity of Hb F according to two different procedures. Kinetic studies of the alkali-denaturation of hemoglobin in single red cells were performed with a slightly modified version of the method described by Matioli and Thorell.2 The erythrocytes were imbedded in a layer 25–35 \( \mu \) thick of polyethylenimine in a reaction chamber previously described and were analyzed in the microspectrophotometer described by Thorell and Akerman.3 A random sample of individual erythrocytes from the peripheral blood of the patient was analyzed. The changes of absorption of HbO_2 at 578 m\( \mu \), after treatment with alkali, were measured for 100 seconds after the start of the reaction on a Speedomax recorder in couple with the microspectrophotometer. The percentage at various times of undenatured Hb was calculated from the spectrophotometric records and plotted semi-logarithmically. The curves showed that every analyzed cell contained a mixture of HbA and HbF and that the alkali denaturation kinetics of HbF are similar to those found in normal fetal erythrocytes.

As the above mentioned procedure is not, at the moment, suited for the quantitative assay of the two hemoglobins, the quantitation of total hemoglobin and of HbF in single erythrocytes was performed according to the methods of Thorell4 and of Matioli et al.5 The ethanol-methanol fixed smears of erythrocytes were photographed on Ilford Chromatic plates No. 30, using monochromatic light at 405 m\( \mu \) and a microscope equipped with an apochromatic condensor, an apochromatic objective 60 x N.A. 0.85 and a compensatory ocular 8x. The total density of the cells, registered with a recording densitometer, was determined by planimetry. The mean extinction values were calculated by referring to the densities of the various steps of a rotating sector represented in each plate. The mean erythrocytic diameter was established by averaging the values of six measurements, taken.

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along diameters of which each formed with the preceding one an angle of about 50 degrees. The total amount of hemoglobin in single cells was thus determined according to Thorell. The same smears were subsequently treated with phosphate-citrate buffer 0.3 M, pH 3.30, for 20 seconds, and the erythrocytes that had been analyzed for the total hemoglobin content were now analyzed for the complement of hemoglobin not extracted by the buffer, by using the same procedure as before. This relatively insoluble hemoglobin fraction is considered to represent almost exclusively HbF.

The amount of HbF \((\alpha_2 \gamma_2)\) found in each cell was subtracted from the total hemoglobin and the amount of HbA \((\alpha_2 \beta_2)\) was obtained from the difference. From these data, the total amount of \(\alpha\-, \beta\-,\) and \(\gamma\-) chains in each cell can be calculated. While the values of the \(\alpha\-\) and \(\gamma\-) chains are presumably correct, the amount of \(\beta\-) chains are slightly overestimated by the contribution of \(\delta\-) chains, since methods for a selective analysis of HbA_2 in single cells are not yet available. The reproducibility of repeated measurements on the same average cell was within ± 2 per cent for total hemoglobin. All cells or cell fragments in a given field of vision were included in the count, although all could not be analyzed spectrophotometrically within tolerable limits of error (± 8 per cent).

RESULTS

Clinical and General Hematologic Findings

The patient, U. S., a 21-year-old woman from Sardinia, was affected with a blood disease clinically diagnosed as thalassemia major (Cooley’s anemia). Analysis revealed a strong anemia, with 6.5 g per cent of hemoglobin. The red cell count was 3.1 x 10^6, hence the M.C.H. (mean cellular hemoglobin) value was 21.0. This last value cannot be considered as precise, in view of the margin of error inherent in red cell counts. The minor hemoglobin component \(A_2(\alpha_2 \delta_2)\) was elevated to 5.8 per cent, according to an electrophoretic analysis on starch gel (Matioli and del Piano). The alkali-denaturation procedure of Jonxis and Huisman yielded for fetal hemoglobin (HbF) the value of 54.5 per cent. By subtraction the value for adult hemoglobin (HbA) should be about 40 per cent. The mean values in \(\mug\) per cell of the individual hemoglobin polypeptide chains is consequently approximately as follows: \(\alpha\-) chain, 10.5; \(\beta\-) chain, 4.2; \(\gamma\-) chain, 5.7; and \(\delta\-) chain, 0.6.

No HbH(\(\beta_4\)) was detected by starch block electrophoresis.

The blood smear (fig. 1) is of a type commonly seen in cases of thalassemia major.

Microspectrophotometric and Related Findings

One hundred cells were examined at random. Twenty of these cells could not be analyzed microspectrophotometrically, either because they were too small and perhaps represented cell fragments (schistocytes), or because the distribution of hemoglobin was too irregular, as for instance in the cell represented furthest to the right of the second row of cells on figure 2. The remaining cells fell into two clearly distinct categories, hypochromic and normochromic cells. Figure 2 represents eight cells selected to show the range of cell size and degree of hemoglobinization, not taking into account “schistocytes.” The total amount of hemoglobin in the cells of the first row is normal in spite of their variation in size; in spite of a similar variation in size, the total hemoglobin in the cells of the second row is very nearly one half of the first row-cell corresponding in diameter and placed just above it.
Fig. 1.—Microphotograph taken at 405 m. (mercury emission line; objective aperture = 0.85) of a blood smear from patient U. S. Note anisocytosis and poikilocytosis commonly found in thalassemia. On the upper part of the picture is the image of the rotating sector used in calibrating the density of the plate.

The results relative to the two cell populations are presented in table 1. The amount of α-chain is calculated as one half the total amount of hemoglobin since all known human and other mammalian hemoglobins are for one-half made up of α-chains. The difference between the amount of γ-chain, established by the method referred to above, and one half the value for total hemoglobin was taken as representing the amount of β- and δ-chains present. The β-chains and δ-chains could not be distinguished on a cellular basis by the technic used, and their respective contributions are therefore pooled.

The proportions of cell populations α and β (table 1), as found in the peripheral blood, probably are not representative of the relative rates of production of these two types of cells, since they may be assumed to differ in mean survival time. Productionwise the normochromic cells may present a very small minority. This should not, however, detract from the significance of their presence.

It is noteworthy that with the exception of the very small cells or cell fragments, which have not been measured and make for less than 20 per cent of the total peripheral cell population, the cell diameters of the thalassemic...
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Fig. 2.—Microphotograph taken at 405 μm (mercury emission line; objective aperture = 0.85) of individual erythrocytes from patient U. S. to show the range of sizes and of degree of hemoglobinization. Each cell of the lower series has a mean diameter comparable with that of the cell placed above it, whereas its hemoglobin content is close to one half that of the cell placed above it.

patient U. S. are nearly normal in both cell groups with respect to their means as well as to their ranges. The small departure from normality observed may not be significant. The “schistocyte” type of cells excepted, there is no clearly significant, and at any rate no marked, microcytosis in this case of “thalassemia.” The range of cell diameters seems to be continuous. Not only is there no correlation between cell diameter and total hemoglobin content of cells, but, independently of this latter parameter, our data do not suggest the existence of two distinct cell populations from the point of view of diameter. The difference in diameter between cell populations a and b is statistically non-significant (t = 0.91, 0.2 < p < 0.4 for 78 degrees of freedom). The quantitative distributions of neither the α-chain (as stated already by the reference to the total hemoglobin content per cell), nor of the β-chain, nor of the γ-chain are correlated with cell diameter.

The values for γ-chain content form but one population over cell groups a and b. A "t-test" comparing the γ-chain content in the two populations yielded t = 1.29, with p ~ 0.20 for 78 degrees of freedom.

DISCUSSION

Thalassemia is defined here as the specific inhibition of a structural hemoglobin chain gene, in the absence of any known alteration of base sequence in that gene, during a period of life in which the gene is normally active.

The apparent unimodality of the distribution of γ-chains is consistent with the view that the stimulation of the γ-chain gene is effected by a quantitative change in a diffusible factor. A change in the rate of production of this factor is assumed to be brought about as a result of a variety of pathologic conditions that put the bone-marrow under "stress." An alternate hypothesis, first proposed by Singer et al.11 according to which the state of activation of the γ-chain gene is directly influenced by the activity of the β-chain gene, is not supported by the present results. The existence of a diffusible factor that
<table>
<thead>
<tr>
<th>Cell Category</th>
<th>Total Cellular Hemoglobin (µg./cell)</th>
<th>α-chain (=MCH*/2) (µg./cell)</th>
<th>β-chain + δ-chain (µg./cell)</th>
<th>γ-chain (µg./cell)</th>
<th>Red Cell Diameter (microns)</th>
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<tr>
<td></td>
<td>Mean</td>
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<td>σ</td>
<td>Mean</td>
<td>Extremes</td>
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<tr>
<td>a</td>
<td>65</td>
<td>14.5</td>
<td>10.0-15.0</td>
<td>2.30</td>
<td>7.3</td>
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<tr>
<td>b</td>
<td>15</td>
<td>31.8</td>
<td>28.0-35.0</td>
<td>2.10</td>
<td>15.9</td>
</tr>
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Normal subject, mean values per total peripheral cell population (from literature)
29† 14.5 14.3 0.2 7.65 6.1-8.6†

*M.C.H. = mean cellular hemoglobin.
†Cf. Wintrobe.‡
‡Ambs.§
acts on the $\gamma$-chain synthesizing system would of course not preclude the possibility of a differential responsiveness of this system in different cell populations and under different circumstances, whereby the increase in $\gamma$-chain production would be made unequal in different cells, as has been claimed to be the case in certain individuals with thalassemia, with sickle cell anemia and with other dysemias (Thompson et al.,\textsuperscript{12} Ballerini et al.,\textsuperscript{13} Shahidi et al.\textsuperscript{14}). But the existence of cases such as the one described here makes a direct linkage between activities of the $\beta$- and the $\gamma$-chain genes very unlikely. The values for the $\beta$-chain are indeed very different in cell populations $a$ and $b$. They vary around two widely separated means. The ratio of these means is 3.8 to 1.0.

The corresponding ratio of the $\alpha$-chains or of total cellular hemoglobin is 2 to 1. Such a ratio, if not due to a numerical coincidence, could be interpreted by the inactivation of one out of two allelic genes.

The inhibition of a gene, namely of a structural gene controlling a hemoglobin polypeptide chain, is characteristic of thalassemia. If the ratio 1 to 2 reflected the thalassemic inhibition of a structural hemoglobin chain gene, we would be dealing with an $\alpha$-thalassemia (cf. Ingram and Stretton\textsuperscript{15}), since this ratio is observed in the $\alpha$-chain. That we do not deal with a case of pure $\alpha$-thalassemia is however indicated by the absence of an excess of $\beta$-chain in the form of HbH (Jones et al.\textsuperscript{16}). Such an excess should be detected when the total $\alpha$-chain production alone is significantly decreased. An excess of $\beta$-chains would be expected to be produced as HbH in 65 per cent of the cells, namely in those where the $\alpha$-chain production is cut to one half its value, the more so as in the normochromic cells where the $\beta$-chain synthesizing system is shown to be able to put out four times more $\beta$-chain per cell than it does in the hypochromic cells.

On the other hand, the presence of a $\beta$-thalassemia in the patient is strongly suggested by the characteristic elevation of the minor hemoglobin component, HbA\textsubscript{2}. The elevation of this component in $\beta$-thalassemia is substantiated in the literature by percentage figures only. But it also obtains, although often to a smaller extent, for absolute mean values per cell of the $\delta$-chain content. We calculate from data in the literature the normal mean absolute quantities per cell of $\delta$-chain range from about 0.2 to 0.4 $\mu$g., as against 0.6 $\mu$g. in the patient U. S. Elevated HbA\textsubscript{2} values have invariably been found in heterozygotes for $\beta$-thalassemia and a $\beta$-chain with a detectable structural anomaly, and these cases leave no doubt about the existence of a specific inhibition of the structural $\beta$-chain gene in $\beta$-thalassemia. Our data show that $\beta$-chain production is inhibited in all cells, strongly in cell group $a$, more weakly in cell group $b$. That the specific inhibition of one $\beta$-chain gene could not alone account for the condition prevailing in cell population $a$ is brought out by the observation, derived from many results to be found in the literature (Zuckerkandl\textsuperscript{*}), that in the presence of an inhibited $\beta$-chain allele, the other is able to compensate for it to a significant extent. A single normal

\textsuperscript{*}In unpublished analyses of data from the literature.
\(\beta\)-chain allele, in the presence of a thalassemic \(\beta\)-chain gene is able to control the production of an average of up to 12 \(\mu\text{g}\) \(\beta\)-chain per cell, as against 7 \(\mu\text{g}\) in an average normal cell, including in both cases a minor contribution from the \(\delta\)-chain.

The question must then be asked whether the present case could be interpreted as one of double heterozygosity for \(\alpha\)- and \(\beta\)-thalassemia. Beside running into other difficulties, such an interpretation would be inconsistent with the observed parallel behavior of the \(\alpha\)- and \(\beta\)-chains which, in a given cell, are either both in the low bracket of values, or both in the high bracket (table 1). A parallel variation from cell to cell of the "penetrance" of both the \(\alpha\)- and the \(\beta\)-thalassemia would point to the intervention of a third factor other than the thalassemia proper. Three factors would be more than necessary to account for the observed situation. While, as a result of the present analysis, it appears that the action of a genic factor other than the specific inhibition of a structural hemoglobin chain gene, a double heterozygosity for \(\alpha\)- and \(\beta\)-thalassemia is improbable.

On the other hand we do not consider that we are dealing with a \(\beta\)-thalassemia homozygote. If we had homozygous \(\beta\)-thalassemia expressed in a mosaic fashion, non-accompanied by a general inhibition of hemoglobin production, the ratio of 2 to 1 of total hemoglobin in the two cell populations could apparently not be interpreted. Our case might conceivably be one of homozygous thalassemia combined with a general inhibition of hemoglobin production in part of the red cells, but this assumption is on the whole unlikely, partly because, judging from the appearance of blood smears, our case does not appear to be exceptional. Also the average value of \(\beta\)-chain per cell over the total erythrocyte population, given above as 4.2 \(\mu\text{g}\), is somewhat high for a thalassemic homozygote (Zuckerkandl*).

The numerical results of this investigation are best accounted for on the basis of a concept previously derived by one of us from an analysis of certain results described in the literature (Zuckerkandl17), and already forecast by Itano.17 One might suppose that the present case is a double heterozygote for \(\beta\)-thalassemia and a mutant of a gene that controls some step essential for the synthesis of all hemoglobin chains. The locus of the latter mutant must be distinct from those of the structural hemoglobin-chain genes and may be called the panglobinopenia locus. The action of the panglobinopenia gene might be thought to be distributed according to the mosaic pattern that is observed. We are thus led to consider that the observed ratio of 1 to 2 does not characterize the activity of the \(\alpha\)-chain synthesizing system specifically, but total cellular hemoglobin production.

The question as to which step in hemoglobin synthesis is inhibited in panglobinopenia can of course receive only a speculative answer at the present time. Since it has been seen that the inhibition probably does not concern protein synthesis in general, it is possible that heme synthesis is involved. This might explain the disturbance in heme synthesis that characterizes

*In unpublished analyses of data from the literature.
"thalassemia" according to Bannerman et al. (cf. Bannerman,18 p. 107). The disorder might really be characteristic of panglobinopenia.

In those cells where the panglobinopenia mutation may be considered not to be manifest, namely in the normochromic cell population b, the condition is compatible with a heterozygous β-thalassemia. The production of β-chain reaches in these cells a relatively high mean level, 11.1 μg./cell, that does however not exceed the potential abilities, as known from numerous other cases, of average β-chain synthesis per cell under the control of one single β-chain gene. Some contribution from the supposedly inhibited (thalassemic) β-chain gene may also occur in the establishment of the figure of 11.1 μg. The β-chain level even in cell population b could thus be attributed to the activity of essentially one single β-chain gene. Therefore the specific inhibition of the β-chain gene, in contradistinction to the panglobinopenia condition, might not be distributed according to a mosaic pattern and might be effective in all cells.

This however need not be so. It is equally possible that cell population b contains two alleles of the β-chain gene that display normal activity at the genic level. The reduction in cell population b of the total amount of β-chain per cell in comparison with red cells of a normal adult would then be due exclusively to a competition between a product derived directly or indirectly from the β-chain genes and an equivalent product from the γ-chain genes for a common acceptor present in limiting amounts. This competition would arise as a result of the stimulation of the γ-chain gene in all cells, and this stimulation, as suggested before, might be attributed to a change in concentration of a diffusible factor, a change brought about by the condition of "stress" that prevails in particular as a result of the cellular fragility of cell population a. The unimodal distribution of γ-chain production that is observed in at least 80 per cent of the peripheral cells may be assumed to reveal a unimodal stimulation of the γ-chain gene in these cells.

The double heterozygote resulting from the combination of a panglobinopenia and a thalassemia gene in one individual is considered to be able to give rise to the clinical picture of thalassemia major. As to homozygotes for the panglobinopenia gene, an unknown proportion of such cases may be lethal at an early stage.

Because of the existence in our case of two erythrocyte populations sharply distinct from the point of view of hemoglobin production, but not of cell size, panglobinopenia and perhaps also thalassemia may belong genetically to the type of conditions (Muller19) that have been described as variegation (Schultz20). This is a type of position effect that results in somatic mosaicism. Mosaicism seems to originate most commonly from the translocation of a gene from its normal position to the neighborhood of a heterochromatic region (c.f. Lewis21). In the case of a heterozygote for a sex-linked gene mosaicism apparently can also be brought about in the mammalian female by the random inactivation of either the paternal or the maternal X-chromosome in different cell lineages (Lyon,22 Beutler et al.23). However, inasmuch as blood smears from many cases of "thalassemia major" appears
qualitatively indistinguishable from ours and therefore suggest that we do not deal with an exceptional case, the mosaicism observed in our case is probably not sex linked.* One may wonder whether pangeninopenia and perhaps also thalassemia are attributable to translocations of one or more autosomal genes, or to inversions of a group of autosomal genes. By allowing one to involve more than one gene, the hypothesis would account for the multiple effects of the thalassemic condition on the red cell which, according to some authorities, it is difficult to ascribe to the pleiotropic effect of a single gene mutation. In this connection one must however bear in mind that some conditions likely to be ascribable to a mutational event relating exclusively to structural hemoglobin genes simulate hematologically a thalassemia, such as the Hb Lepore mutation ([Jonxis]²⁵).

The process whereby the genes subject to variegation are kept preferentially in a state of synthetic inactivity would be assumed not to be operative at the start of embryonic development and to come into play during its course, although probably at an early time. Some erythrocyte precursor cells, affected by this process, would give rise to the lineage from which normochromic cells are derived during the remainder of the individual's life time. An alternate hypothesis is less likely, namely that all red cell precursors are genetically identical, but in a metastable state, so that local variations in the conditions prevailing in the hematopoietic tissue during adult life will orient the development either toward cells of type a or cells of type b. If such were the case, one would expect significant variations in the proportions of the different cells during the evolution of the disease, to be reflected in the MCH value as well as in the overall proportion of HbF and HbA₂ present in the blood. These values are however relatively constant and characteristic of a given individual (Matioli and del Piano)⁷. The assumption of the existence of red cell precursors of more than one type in thalassemia is in accord with observations that suggest indirectly the development in independent clusters within the hematopoietic tissue of red cells differing in the amount of hemoglobin they contain. This is indicated by the distribution of hemosiderin granules. Since the cellular production of hemosiderin has been shown by one of us (Matioli)²⁶ to be inversely proportional, at least in semiquantitative terms, to the production of hemoglobin, the hemosiderin granules in red cell precursors can be taken as markers for their potentialities with respect to hemoglobin production. In thalassemic patients the quantity of ferritin granules per cell varies from cell cluster to cell cluster and is relatively stable within any given cell cluster. A bimodality of distribution is suggested here also (Matioli)²⁶.

The proportion of erythrocyte precursor cells affected by the variegated genic inhibition may vary from individual to individual, perhaps as a result of a variation in timing of the inactivation in relation to embryonic develop-

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* A striking example of mosaicism with respect to total hemoglobin production is offered by pictures of blood smears from patients with acquired achreptic anemia (Dacie et al.²⁴), and this condition is not sex linked.
ment, and this may account for a good deal of the variability of the "thalassemic" syndrome. It may even account in certain cases for the difference clinically characterized as that between thalassemia minor and thalassemia major, a distinction which on purely clinical grounds is quite illegitimate to interpret genetically.

Evidently the tentative interpretation of the present results remains entirely hypothetical. It will now be confronted with further results to be obtained through the application of the same methods to various cases of "thalassemia."

Summary

Microspectrophotometric hemoglobin determinations on individual red cells from the peripheral circulation of a patient clinically characterized as a case of thalassemia major show the presence of two main cell populations, one normochromic and one hypochromic, with a ratio of total hemoglobin per cell of 2 to 1, a subnormal \( \beta \)-chain production in all cells, at mean levels that are in a ratio of 4 to 1 in the two cell populations, and a strongly stimulated \( \gamma \)-chain production in both cell populations with no bimodality of distribution. The mean cellular content of \( \delta \)-chain is augmented. The ratios of 2 to 1 for total hemoglobin production is explained on the basis of the hypothesis that the case is one of heterozygosity for a \( \beta \)-thalassemia gene and a panglobinopenia gene with a mosaic condition realized at least in the case of the panglobinopenia gene. "Panglobinopenia" is described as a condition leading to a general inhibition of hemoglobin synthesis, perhaps through an inhibition of heme synthesis. To account for the observed cellular mosaic, it is proposed that panglebinopenia and perhaps thalassemia are examples of the well known phenomenon of variegation. This interpretation is now to be tested by applying the same methods to the study of further cases of thalassemia patients.

Summary in Interlingua

Determinaciones microspectrophotometric de hemoglobina in erythrocytos individual ab le circulation peripheric de un patiente clinicamente classate como un caso de thalassemia major demonstra le presentia de duo principal populationes de cellulas—le un normochromic, le altere hypochromic—con un proportion de hemoglobina total per cellula de 2 a 1, un subnormal production de catenas \( \beta \) in omne le cellulas con valores medie in le duo populationes a un proportion de 4 a 1, e un fortemente stimulate production de catenas \( \gamma \) sin bimodalitate del distribution. Le valor medie del contento cellular de catenas \( \delta \) es augmentate. Le proportion de 2 a 1 in le production de hemoglobina total es explicate a base del hypothese que le caso es un de heterozygoticitate pro un gen de thalassemia \( \beta \) e un gen de panglobinopenia con un condition de mosaico realisate al minus in le caso del gen de panglobinopenia. "Panglobinopenia" es describite como un condition resultante in un inhibition general del synthese de hemoglobina, possibilemente per un inhibition del synthese de hem. Pro explicar le observate mosaico cellular, il es stipulate que
panglobinopenia e forsan thalassemia es exemplos del ben-cognoscite pheno-
meno de variegation. Iste interpretation nunc debe esser testate per applicar
le mesme methodos a casos additional de patientes con thalassemia.

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Gastone Matioli, M.D., Assistant Professor, Department of Microbiology, University of Southern California School of Medicine, Los Angeles, Calif.

Emile Zuckerkandl, Ph.D., Professor, Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, Calif. Present address: University of Montpellier, France
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