Intraerythroblastic Instability of Hemoglobin \(\beta^+\) (Hgb H)

By PHAEDON FESSAS AND XENOPHON YATAGHANAS

SPONTANEOUS PRECIPITATION of the excess \(\alpha\)-chains is a characteristic feature of the erythroblasts in severe and intermediate \(\beta\)-thalassemia.\(^1\,^2\) In hemoglobin H disease (\(\alpha\)-thalassemia intermedia) precipitation of the excess \(\beta\)-chains in vivo has been observed in the erythrocytes of peripheral blood only following splenectomy\(^3\,^4\,^5\,^6\); the possibility of intracellular precipitation occurring at the bone marrow stage has apparently not been investigated so far.

PATIENTS AND METHODS

Three patients with hemoglobin H disease were available for this study. The essential clinical, hematological and electrophoretic data are given on Table 1. Patient 3 was in late pregnancy and had a megaloblastic bone marrow, due probably to folic acid deficiency; she received folic acid and had a normal delivery. This patient was reexamined several months later but permission for another marrow puncture was not given.

For microscopy and staining, essentially the same methods, as discussed in a previous publication,\(^1\) were used. The method of incubation with brilliant cresyl blue was applied to demonstrate the formation of typical hemoglobin H inclusion bodies in vitro.\(^7\) Vital staining with methyl violet was used for demonstrating intracellular hemoglobin precipitates which had formed spontaneously in vivo. The vital staining of bone marrow aspirates collected in ACD solution was improved by applying mild centrifugation to remove excess liquid whenever necessary. A further improvement consists in adding the dye as a 1 percent solution in AB plasma instead of saline, in which way cellular morphology is better preserved.

Caryometric measurements were performed on the methyl violet stained marrow preparations with the Leitz-Ortholux microscope equipped with an appropriate calibrated micrometer scale. Over 500 consecutive nucleated cells were measured on each marrow; the inclusions were characterized always by the same observer as precisely as possible and placed in one of three classes: class I for small coccoid forms or diffuse staining, class II for medium sized inclusions and class III for the large precipitates. Parallel counts of inclusion carrying red cells of the bone marrow preparations and peripheral blood were also done.

RESULTS

The peripheral blood preparations of the two patients with intact spleen presented only occasional cells with methyl violet positive inclusions; these were generally small and varied from one to four per cell, rarely more (Fig. 1...
<table>
<thead>
<tr>
<th>Case</th>
<th>Sex, Age</th>
<th>Clinical features</th>
<th>Transfusions</th>
<th>Hemoglobin concentration</th>
<th>Mean corpuscular volume, %</th>
<th>Mean corpuscular hemoglobin, per 100 WBC</th>
<th>Starch gel electrophoresis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M, 24</td>
<td>Mild pallor, subjectively spleen 6 cm. b.c.m.</td>
<td>Never</td>
<td>12.1</td>
<td>5.64</td>
<td>46</td>
<td>&lt;1</td>
</tr>
<tr>
<td>2</td>
<td>F, 45</td>
<td>Pallor, Splenomegaly 8 cm. b.c.m.</td>
<td>Once during pregnancy</td>
<td>8.0</td>
<td>4.88</td>
<td>29</td>
<td>&lt;1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.8</td>
<td></td>
<td>A(_2) decreased, F traces</td>
</tr>
<tr>
<td>3A</td>
<td>F, 24</td>
<td>Pallor, mild mongoloid facies, subjectively spleen 3 cm. b.c.m.</td>
<td>Several</td>
<td>8.2</td>
<td>3.45</td>
<td>30</td>
<td>11.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>A(_2) decreased, F traces</td>
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<tr>
<td>3B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>9.1</td>
</tr>
</tbody>
</table>

**Table 1.**—Clinical and Laboratory Data on Patients Studied

- Case 1: Male, 24 years old, with mild pallor and subjectively spleen 6 cm. b.c.m. No transfusions. Hemoglobin concentration 12.1%, mean corpuscular volume 5.64%, mean corpuscular hemoglobin 46. The starch gel electrophoresis result is <1.

- Case 2: Female, 45 years old, with pallor and splenomegaly 8 cm. b.c.m. Transfused once during pregnancy. Hemoglobin concentration 8.0%, mean corpuscular volume 4.88%, mean corpuscular hemoglobin 29. Starch gel electrophoresis result is <1.

- Case 3A: Female, 24 years old, with pallor, mild mongoloid facies, and subjectively spleen 3 cm. b.c.m. Received several transfusions. Hemoglobin concentration 8.2%, mean corpuscular volume 3.45%, mean corpuscular hemoglobin 30. Starch gel electrophoresis result is 11.2.

- Case 3B: No lab data provided, patient received no transfusions. Hemoglobin concentration 9.1%, mean corpuscular volume 3.81%, mean corpuscular hemoglobin 31. Starch gel electrophoresis result is 10.2.
In agreement with previous reports,\textsuperscript{6} the splenectomized case presented numerous inclusion-carrying red cells (Fig. 1 c); the morphology of these intraerythrocytic precipitates was very variable and the classical round postsplenectomy Heinz-body was rather rare (Fig. 1 d).

The bone marrow preparations of all three patients revealed the presence of many normoblasts containing methyl violet staining precipitates; such were also easily observed in the circulating normoblasts of the splenectomized patient 3. The morphological characteristics of the precipitates are shown on Fig. 2; their size and shape vary greatly, their outline is generally irregular and often they have a filamentous or loose structure resembling to the reticulum. Thus, they differ from the typical round and dense postsplenectomy inclusion reported in the circulating red cells, although they have the same staining properties. The precipitates are seen most commonly close to the nucleus, as one or more spots or, rarely, as a crescent-shaped paranuclear zone; the binucleated megaloblasts of case 3 often had inclusion close to each nucleus (Fig. 2 d). The possibility that we were dealing with an artefact was
Table 2.—Quantitative Data on Intracellular Hemoglobin Precipitation

<table>
<thead>
<tr>
<th>Case</th>
<th>% of BCB inclusions*</th>
<th>Percent of methyl violet staining inclusions in Red cells</th>
<th>Percent of methyl violet staining inclusions in Erythroblasts</th>
<th>Percentage distribution of inclusion carrying erythroblasts</th>
<th>Hemosi-deria</th>
<th>M : E ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>72</td>
<td>&lt;0.2</td>
<td>1.5</td>
<td>49 48 3</td>
<td>++</td>
<td>1:1.5</td>
</tr>
<tr>
<td>2</td>
<td>46</td>
<td>&lt;0.5</td>
<td>7.0</td>
<td>31 52 17</td>
<td>++ +</td>
<td>1:1.4</td>
</tr>
<tr>
<td>3A</td>
<td>16</td>
<td>38</td>
<td>95.4</td>
<td>25 58 17</td>
<td>++ +</td>
<td>1:1.6</td>
</tr>
<tr>
<td>3B</td>
<td>26</td>
<td>66</td>
<td>93.8</td>
<td>Not examined</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*RBC inclusions: Artefactual precipitates of Hgb H after incubation with brilliant cresyl blue. All other data on inclusions refer to precipitates demonstrated by vital staining with methyl violet.
Fig. 2.—Bone marrow preparations stained with methyl violet, from case 2 (a and b) and 3 (c and d); note cluster of inclusion-carrying red cells in 2b.

excluded by observing the cells on fresh, wet or dried, preparations under phase contrast microscopy. Other staining characters of these inclusions, indicative of their hemoglobinic nature, are identical to those of their counterparts found in β-thalassemia¹ and will not be repeated here. Quantitative data on the intraerythroblastic precipitates are given in Table 2. Since the assessment of hemoglobinization by this vital stain is not very accurate, we used the nuclear diameter as an index of cellular maturity. The progress of formation of inclusions in the nucleated cells of the bone marrow is shown on Fig. 3. The findings in patients 1 and 2 were rather similar; precipitation was visible at a nuclear diameter around 7 μ, which normally corresponds to the early polychromatophilic stage, and the phenomenon became more intense at smaller nuclear diameters. The highest percentage of inclusion-carrying cells and the larger precipitates were recorded in the more mature normoblasts. Case 1, clinically and hematologically the mildest of the three, had both the lowest percentage of inclusion-carrying normoblasts and the smallest proportion of class III inclusions. The findings on case 2 are shown in more detail on Fig. 4. In case 3 the distribution of affected normoblasts was somewhat different:
Fig. 3.—Evolution of hemoglobin precipitation in nucleated erythroid cells of bone marrow. The total of inclusion-carrying cells expressed as percent of all nucleated red cells in each class of nuclear diameter.

Fig. 4.—Evolution of hemoglobin precipitation and erythroblastic maturation in case 2, expressed in absolute numbers.
precipitation begins in cells with larger nuclei and the entire caryometric curve is spread and flattened, probably because of the megaloblastic transformation.

**DISCUSSION**

It is generally assumed, that precipitation of \( \beta_4 \) is to a great extent a function of cellular age, occurring after delivery of the erythrocytes. Our findings, without questioning this process, indicate that precipitation of \( \beta_4 \) starts intramedullary. The typical large postsplenectomy inclusion which apparently consists of denatured Hgb H, would then constitute the end stage of a continuous process. Although chemical analysis of the intraerythroblastic inclusions presented here has yet to be obtained, it is logical to conclude that they consist of denatured \( \beta \)-chains.

Since precipitation of Hgb H starts early, it is necessary to explain the large discrepancy between the percentages of inclusion-carrying cells of the bone marrow and of the peripheral blood in the patients with intact spleen. Erythropoiesis in the Hgb H disease has been considered as effective, as judged from the absence of retention of Fe\(^{59}\) over the marrow. On the other hand, all studies unanimously report a low red cell iron utilization, from 40-75 percent. This could be due to dilution of the label into a large intraerythroblastic pool of iron, followed by rapid removal of siderotic granules. Alternately, the low iron utilization can be ascribed to the early precipitation of some Hgb H. Probably most cells with inclusions reach the circulation, where some are removed in toto while others are freed of their inclusions by the splenic pitting function. This sequence of events is supported by the short survival of Hgb H delivered to the circulation in the soluble form.

Evidence in favor of delivery of cells with preformed inclusions can be found in the work of Malamos et al.: injection of Fe\(^{59}\) is followed by an early intense rise of radioactivity over the spleen, while with Cr\(^{51}\) tagged red cells no simultaneous preferential uptake by this organ is recorded. However these technics do not permit to distinguish between splenic uptake of siderotic granules or of hemoglobin precipitates. The data of Gabuzda et al. can be used in support of an early precipitation of \( \beta \)-chains: after incubation of blood or marrow with C\(^{14}\)-glycine the ratio of specific activities of Hgb H to A ranged between 4 and 7.5; however the Hgb H/A ratio obtained by in vivo studies on the same patients was only 2 to 3 when the label first appeared in the circulating cells and continued to decline.

The inherent instability of \( \beta_4 \) is probably the basic cause for its early precipitation, but not necessarily the only one. High initial concentration of \( \beta_4 \) could be one contributing factor; the cases of Rigas et al., averaging 40 percent of Hgb H in the circulating cells, indicate that presumably higher concentrations at earlier stages are not of necessity more deleterious. The question arises whether in our patients, case 3 in particular, we are dealing with a minor structural alteration in the \( \beta \)-chain, undetectable by current means, which leads to a different pattern of polymerization and precipitation:

*The possibility that the precipitates are due to extremely unstable, structurally abnormal, \( \alpha \)-chains is excluded in case 3 on genetic evidence.
such hypothesis has been advanced by Ingram and Stretton on different
grounds.\textsuperscript{10} Before accepting this explanation, it is necessary to consider
environmental factors. Younger cells generally provide a more efficient protection
of tetrameric hemoglobin; the normoblasts, however, differ structurally and
functionally from anucleate cells and are situated in another microenvironment.
It is possible that in these cells labilization of uncombined chains or of easily
dissociable molecules is enhanced by certain factors; their effect may vary from
cell to cell, from patient to patient and at various times and may be responsible,
in part, for the reported wide range of percentages and the fluctuations of
Hgb H, as well as for its unequal distribution over the red cell population.
Simple prolongation of the sojourn of erythroblasts in the marrow, as in our
case with megaloblastosis, may provide opportunity for additional precipita-
tion.

Since so many factors determine the ultimate percentage of soluble Hgb H
in the circulation, extrapolations to the level of hemoglobin synthesis should
be done with caution. The wide range of Hgb H—or even its complete ab-
sence—may well be due to different degrees of the $\alpha$-chain deficiency or of the
"efficiency" for $\beta$-chain synthesis, be they determined genetically or otherwise;\textsuperscript{6}
equally well, the variable levels may reflect differences in the intensity of both
intraerythroblastic and postmedullary precipitation of $\beta$-chains and of its equiv-
alent variants. In this connection, the failure to detect $\beta$-tetramers in $\alpha$-thalas-
semia disease combined with Hgb E trait has been considered as evidence
that $\alpha$-thalassemia genes are similar to regulatory genes, affecting synthesis of
$\beta$-type chains as well.\textsuperscript{11} Such departure from the prevailing view on the specific action of thalas-
semia genes will be necessary when any intracellular and, more precisely, intraerythroblastic precipitation of hemoglobin or of its sub-
units has been excluded.\textsuperscript{12}

The present observations demonstrate another basic parallel between $\alpha$ and
$\beta$-thalassemia,\textsuperscript{12,13} namely intraerythroblastic precipitation of unpaired hemo-
globin chains. In homozygous $\beta$-thalassemia however, the phenomenon is more
pronounced but less complex: precipitation of the surplus $\alpha$-chains is nearly
complete at the erythroblastic stage and only minute amounts of the soluble
$\alpha$-chains are left over to form new precipitates after delivery of the erythro-
cytes.\textsuperscript{14}

\textbf{Summary}

The bone marrow of three cases of hemoglobin H disease was examined by
vital staining with methyl violet. Intraerythroblastic precipitation of hemoglo-
in, presumably $\beta$-chains, was observed in varying degree in all three cases.

\textsuperscript{6}The medical literature contains one case of symptomatic $\alpha$-thalassemia in which Hgb
$\gamma_4$ but no Hgb H were found.\textsuperscript{16} This case, reported at a time the concept of thalas-
semia was not yet formulated, was reexamined by us through the kindness of Dr. Zannos-Mariolea;
hemoglobin H and BCB-inclusion bodies could be demonstrated, but $\beta_4$ was somewhat
less than $\gamma_4$. The comparable case of an Italian girl with persistence of Hgb $\gamma_4$,\textsuperscript{16} apparently
contained minute amounts of $\beta_4$; the morphology of routinely stained marrow normoblasts,
as described by the authors, is not incompatible with intracellular precipitation of hemo-
globin.
INTRAERYTHROBLASTIC INSTABILITY OF HEMOGLOBIN $\beta_4$  

The process has been studied in relation to erythroblastic maturation and the occurrence of precipitates during early stages of hemoglobin synthesis was demonstrated. The observations are discussed in relation to present-day knowledge concerning hemoglobin $\beta_4$ and the $\alpha$-thalassemias.

SUMMARIO IN INTERLINGUA

Le medulla ossee de ties subjectos con morbo de hemoglobina H esseva examinate per tincturation vital con violetta methylic. Percipitation intraerythroblastic de hemoglobina-persumitemente catenas 1-esseva observate in omne le casos ben que vane grados de intensitate. Le processo esseva studiate in relation con le maturation erythroblastic e le occurrentia de precipitationes durante le precoce stadis del synthese de hemoglobina esseva demonstrate. Le constatationes es commentate con referentias al currente cognoscentias de hemoglobina $\beta_4$ e le thalassemias $\alpha$.

ACKNOWLEDGMENTS

We thank Dr. (Mrs.) Papaspyrou-Zonas for her kind assistance in the study of case 1 and Dr. K. Sofroniadou for generous help in the study of case 3.

REFERENCES

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