Observations on the Levels of Hb A\textsubscript{2} in Patients With Different \(\beta\)-Thalassemia Mutations and a \(\delta\) Chain Variant

By J.F. Codrington, H-W. Li, F. Kutlar, L-H. Gu, M. Ramachandran, and T.H.J. Huisman

Hb A\textsubscript{2} and its variant B\textsubscript{2} \((\alpha_2\delta_1\text{A13Gly} \rightarrow \text{Arg})\) were quantitated in the blood of subjects with three different types of \(\beta\)-thalassemia and with the \(\delta\)-B\textsubscript{2} anomaly in cis or in trans to the \(\beta\)-thalassemia determinant. In one family, the \(\delta\)-B\textsubscript{2} mutation was in cis to a newly discovered codon 47 \((+\text{A})\) frameshift. The levels of Hbs A\textsubscript{2} and B\textsubscript{2} were nearly the same and approximately 70\% higher than those in simple Hb B\textsubscript{2} heterozygotes. In two additional families, the \(\delta\)-B\textsubscript{2} variant was in trans to either a deletional \(\beta\)-thalassemia (1,393 bp) involving part of the \(\beta\)-globin gene and part of the \(\beta\)-globin gene promoter, or to the \(-88\ C \rightarrow T\) promoter mutation. In both instances, the Hb B\textsubscript{2} level was increased by approximately 80\%, but the Hb A\textsubscript{2} level was increased by approximately 270\% and 200\%, respectively. These data indicate two mechanisms that will cause an increase in \(\delta\) chain production. One is consistent with a general mechanism concerning the relative excess of \(\alpha\) chains in \(\beta\) chain deficiencies which will combine with \(\delta\) chains to form variable levels of Hb A\textsubscript{2} dependent on the severity of the \(\beta\) chain deficiency. The second concerns the loss of \(\beta\)-globin gene promoter activity, perhaps by an absence of (or decreased) binding of specific protein(s) to this segment of DNA and a concomitant increase in \(\delta\)-globin gene promoter activity in cis.

The increase in the level of Hb A\textsubscript{2} in \(\beta\)-thalassemia (thal) heterozygotes was discovered in 1957 when the percentage of Hb A\textsubscript{2} was found to be approximately twice that in normal adults. More recent studies have indicated that the relative increase depends on the type of \(\beta\)-thal allele that is present. For instance, carriers of some mild types of \(\beta\)-thal, such as those caused by the C\textsuperscript{-G} mutations in the polyadenylation site, have only slight increases in their Hb A\textsubscript{2} percentages, while heterozygotes for a silent type of \(\beta\)-thal due to the C\textsuperscript{-T} mutation at the 5\' segment of the \(\beta\)-globin gene promoter have nearly normal Hb A\textsubscript{2} levels. These observations are consistent with the concept that \(\alpha\) chains combine much more readily with the \(\beta\) chain of Hb A than with the \(\delta\) chain of Hb A\textsubscript{2}, and that in conditions with variable \(\beta\) chain deficiencies, the increase in Hb A\textsubscript{2} level should vary accordingly. However, there are exceptions to this rule; for instance, persons with a heterozygosity for either one of two mild promoter mutations (namely, C\textsuperscript{-G} at position 88 or A\textsuperscript{-G} at position 29, which are common among blacks) have Hb A\textsubscript{2} values at least as high as those observed among heterozygotes for the more severe \(\beta\)-thal alleles, like the C\textsuperscript{-T} mutation at codon 39 or the G\textsuperscript{-A} mutation at IVS-I-110 present in Mediterranean patients. Furthermore, \(\beta\)-thal heterozygotes with a \(\delta\) chain variant like \(\delta\)-B\textsubscript{2} or \(\delta\)16\textsuperscript{(A13)Gly} \rightarrow \text{Arg}\) in cis or in trans to the thalassemia mutation have similar amounts of Hb A\textsubscript{2} and the Hb A\textsubscript{2} variant, indicating that the increased Hb A\textsubscript{2} synthesis in \(\beta\)-thal is derived from \(\delta\) genes both in cis and in trans to the \(\beta\)-thal mutation.

Unusually high Hb A\textsubscript{2} levels of 7\% to 9\% have been found in \(\beta\)-thal heterozygotes with a deletional defect that involves the 5\' segment of the \(\beta\)-globin gene and part of the \(\beta\)-globin gene promoter. The deletions concern a 292-bp deletion observed in a Turkish family, a 1,393-bp deletion observed among blacks, and the much larger 4,237-bp deletion seen in members of a Czechoslovakian family, all types share the loss of the 5\' \(\beta\) promoter. We recently detected a \(\beta\)-thal heterozygote from Surinam who had the 1,393-bp deletion and a Hb B\textsubscript{2} heterozygosity and who exhibited disproportionate levels of Hb A\textsubscript{2} and Hb B\textsubscript{2}. This observation prompted us to reevaluate some members of two families with both \(\beta\)-thal and Hb B\textsubscript{2} heterozygosities who were described over 25 years ago.

MATERIALS AND METHODS

Blood samples. Samples from three members of family K\textsuperscript{-} and from four members of a second black family (family M) were collected in The Netherlands (two samples) and Surinam (five samples) and shipped by air to Augusta, GA. Blood samples from one black male of family R, his 6-year-old twin daughters, and from a fourth, unrelated adult black female were obtained locally. Collection was in vacutainers with ethylenediamine tetraacetate as anticoagulant. Informed consent was obtained.

Methods. Hematologic data were collected with an automated cell counter. Hb F was quantitated by cation exchange high performance liquid chromatography (HPLC) using the PolyCAT (Columbia, MD) column system. Hb A\textsubscript{2} and Hb B\textsubscript{2} were quantitated by the same PolyCAT HPLC procedure and by the analytic diethylaminoethyl (DEAE)-cellulose procedure with Trisk KCN-HCI developers and a pH gradient; a similar method was used in the earlier publications describing some of these individuals. DNA was isolated from white blood cells with the method described by Poncz et al. Identification of the B\textsubscript{2} variant (\(\delta16\text{(A13)Gly} \rightarrow \text{Arg}\)) was by hybridization of amplified DNA with probes specific for a G\textsuperscript{-}C mutation in codon 16 of the \(\delta\)-globin gene. A similar procedure was used to identify one of the \(\beta\)-thal mutations; this methodology has been described in previous publications. The approximately 1.4-kb deletion present in the third family was detected by gene mapping as shown earlier. The mutation in the third family was new and was identified through sequencing of amplified DNA. It was confirmed by hybridization of amplified DNA with a specific, radiolabeled, oligonucleotide probe.

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Submitted March 13, 1990; accepted May 21, 1990.

Supported by US Public Health Service research grants no. HLBB-05168 and HLB-41544.

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RESULTS

The three families and their β-thal types. Family K is of Dutch-Surinam origin and was first described in 1961. Fourteen members with a β-thal heterozygosity also had a heterozygosity for Hb B2 or α2δ16(A13)Gly → Arg; no normal members carried the Hb B2 variant and no β-thal heterozygote was without it. This observation was explained by assuming that the two abnormalities were linked and occurred on the same chromosome. The average values for Hb A2 (2.0%) and Hb B2 (1.9%) were only slightly less than the average Hb A2 value (2.2%) for 12 normal family members (measured by carboxyl-methyl-cellulose chromatography). Nearly 30 years later, it was possible to obtain blood samples from three β-thal heterozygotes (listed in Table 1). The β-thal mutation in this family had not been observed before, and was identified through sequencing as a frameshift in codon 47 (+A), which was confirmed by hybridization of amplified DNA with appropriate probes (Fig 1).

Family R is a black family from central Georgia who were described in 1963 because Hb C, β-thal, and Hb B2 occurred in different combinations. One subject, E.R., had the β-thal and Hb B2 heterozygosis; family studies showed that the two anomalies were not linked and occurred on opposite chromosomes. Subject E.R. had 1.85% Hb B2 and a rather high Hb A2 level of 2.7%, while his mother, with a simple Hb B2 heterozygosity, had 1.15% Hb B2 and 1.25% Hb A2. He was reevaluated 26 years later; his twin daughters, 6 years of age, were found to have a simple Hb B2 heterozygosity. The β-thal mutation in this family was the second most common type found in blacks, i.e., the C → T mutation at nucleotide −88 of the β-globin gene promoter; details of this characterization are not presented.

Family M, from Surinam, consisted of parents and two children. The father had an Hb B2 trait and a β-thal heterozygosity, the mother an Hb S trait, one child Hb S-β-thal, and the other child Hb S trait combined with a Hb B2 heterozygosity. The β-thal was identified as the 1,393-bp deletion with a 5’ end point approximately 600 bp 5’ to the Cap site and a 3’ end point approximately 500 bp from the 5’ splice junction of IVS-II. Details of the study of family M will not be presented.

The mutation of G → C in codon 16 of the β-globin gene leading to the synthesis of the δ chain of Hb B2 (δ16(A13)Gly → Arg) was confirmed for all three families through hybridization of amplified DNA with 32P-labeled oligonucleotide probes.

The percentages of Hb A2 and Hb B2. Quantitation was with two procedures. The DEAE-cellulose chromatographic method resembled that used in the older experiments; it had the disadvantage of a decreased recovery of Hb B2, particularly in somewhat older red blood cell lysates. The data obtained with the PolyCAT HPLC method were highly reproducible, even after storage of the samples at 4°C for an extended period of time. The percentages determined with both procedures are listed in Table 1; although the absolute values given for some samples in the two groups differ considerably, the relative quantities for Hb B2 and Hb A2 (listed as 100.B2/total) were similar.

The percentages of Hb A2 and Hb B2 in the three members of family K with the codon 47 (+ A) frameshift were nearly the same, confirming previously published results. The average PolyCAT values of 2.41% and 2.49% were approximately 70% higher than the average values of 1.40% and 1.49% observed for two Hb B2 heterozygotes. Subject E.R., with the −88 (C → T) mutation, averaged 2.55% for Hb B2 and 4.48% for Hb A2; this corresponds to approximately 80% and 200% increases over the levels in the Hb B2 heterozygotes. The total value of 7.03% for δ chain containing hemoglobin types was high; the average value for Hb A2 in 10

Table 1. The Levels of Hb A2 and Hb B2 in Hb B2 Heterozygotes With and Without an Additional β-Thal Heterozygosity

<table>
<thead>
<tr>
<th>Subject*</th>
<th>Sex/Age (y)</th>
<th>DEAE-Cellulose Chromatography</th>
<th>PolyCAT HPLC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>B2 (%)</td>
<td>A2 (%)</td>
</tr>
<tr>
<td>Family K (Hb B2 in cis, β-thal mutation, codon 47 (+A))</td>
<td>S.K. (IV-1)</td>
<td>F/36</td>
<td>1.98</td>
</tr>
<tr>
<td></td>
<td>H.R. (III-4)</td>
<td>M/64</td>
<td>2.52</td>
</tr>
<tr>
<td></td>
<td>E.C.R. (III-2)</td>
<td>F/60</td>
<td>1.71</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td></td>
<td>2.07</td>
</tr>
<tr>
<td>Family R (Hb B2 in trans, β-thal mutation, −88 (C → T))</td>
<td>E.R. (II-2)</td>
<td>M/39</td>
<td>2.17</td>
</tr>
<tr>
<td></td>
<td>Second collection</td>
<td></td>
<td>2.46</td>
</tr>
<tr>
<td>Family M (Hb B2 in trans, β-thal mutation, 1,393-bp deletion)</td>
<td>L.M.</td>
<td>M/54</td>
<td>1.77</td>
</tr>
<tr>
<td>Controls (β-thal absent)</td>
<td>S.B.</td>
<td>F/8</td>
<td>1.36</td>
</tr>
<tr>
<td></td>
<td>L.B.</td>
<td>F/6</td>
<td>1.29</td>
</tr>
<tr>
<td></td>
<td>D.C.</td>
<td>F/37</td>
<td>1.24</td>
</tr>
<tr>
<td></td>
<td>Average</td>
<td></td>
<td>1.30</td>
</tr>
<tr>
<td></td>
<td>D.M.</td>
<td>F/13</td>
<td>1.51</td>
</tr>
</tbody>
</table>

Abbreviation: ND, not determined.

*Numbers between parentheses identify the numbers in the pedigrees as published in 1961 and 1963. The controls S.B. and L.B. are the twin daughters of E.R. of family R. The control D.M., who is the daughter of L.M., has an additional Hb S heterozygosity; the value for Hb A2 with the polyCAT HPLC procedure may be slightly too high because of contamination with a minute quantity of a modified Hb S.
\(\beta\)-thal heterozygotes with the same mutation was 5.4% ± 0.4%, with a range of 4.5% to 6.6% (unpublished data). The level of Hb B\(_2\) in L.M. of family M, with the 1,392-bp deletion, was 2.5% and that of Hb A\(_2\) was 5.47%, an increase of approximately 80% and of 270% over the values for Hb B\(_2\) and Hb A\(_2\), respectively, in the Hb B\(_2\) heterozygotes. The level of 7.98% for \(\delta\) chain containing Hb types is similar to values observed for other patients with the same deletion.\(^6,10,13\) The values for Hb A\(_2\) and Hb B\(_2\) in the one nonthalassemic member of this family who also has an Hb S heterozygosity were nearly the same.

**DISCUSSION**

The results obtained in this study, summarized in Table 1, suggest the following. First, simple Hb B\(_2\) heterozygotes have nearly equal levels of Hb A\(_2\) and Hb B\(_2\), indicating that the G → C substitution at codon 16 of the \(\delta\) gene (Gly → Arg in the \(\delta\) chain) does not affect the relative synthesis of this variant chain and, thus, the presence of this \(\delta\) gene marker offers an opportunity to study the relative formation of Hb A\(_2\) derived from \(\delta\) chains in cis or in trans to a \(\beta\)-thal mutation. The one child of family M with Hb B\(_2\) and Hb S heterozygosities also had closely similar Hb A\(_2\) and Hb B\(_2\) values. Second, the nearly equal values for Hb A\(_2\) and Hb B\(_2\) in family K with the frameshift at codon 47 (+A) confirm earlier data\(^1\) and suggest that mutations within the \(\beta\)-globin gene do not influence the relative synthesis of \(\delta\) chains derived from \(\delta\) genes in cis or in trans to the \(\beta\)-thal gene but only the total Hb A\(_2\) formation. Third, the high level of Hb A\(_2\) of nearly 5.5% in a subject with the deletional \(\beta\)-thal who has a \(\delta\)-B\(_2\) mutation in trans and a Hb B\(_2\) level of 2.5% indicates that the excess Hb A\(_2\) is derived from \(\delta\) chains in cis to the \(\beta\)-thal deletion. Finally, the high level of Hb A\(_2\) (nearly 4.5%) in a patient who had the −88 C → T mutation and the Hb B\(_2\) mutation in trans (the Hb B\(_2\) value was 2.5%) showed that this mutation in the CACC box of the 5′\(\beta\) promoter increases Hb A\(_2\) formation from \(\delta\) chains in cis to this \(\beta\)-thal mutation. There are considerable differences between the Hb A\(_2\) and Hb B\(_2\) percentages found for this person; in 1963, at the age of 11 years, the values were Hb A\(_2\) 2.70% and Hb B\(_2\) 1.80% and, in 1990, at the age of 39 years, the average Hb A\(_2\) and Hb B\(_2\) values by DEAE-cellulose chromatography were 4.57% and 2.32%, respectively. The considerably lower values found in the earlier study might be due to a coexisting iron deficiency, although this possibility was not evaluated at that time.

These data suggest that at least two mechanisms can be responsible for higher levels of Hb A\(_2\) in \(\beta\)-thal heterozygotes. One general mechanism concerns the relative excess of \(\alpha\) chains in \(\beta\) chain deficiencies that is present in all types of \(\beta\)-thal, albeit at different levels dependent on the type of \(\beta\)-thal. The second mechanism appears to be loss of \(\beta\)-globin gene promoter activity. Popovich et al\(^1\) were the first to suggest that the transcription of the \(\delta\)-globin gene is affected by loss of the 5′\(\beta\) promoter, perhaps because the 5′\(\beta\) and 5′\(\beta\) promoters are influenced by the same 3′\(\beta\) enhancer. The data presented here support this hypothesis and suggest that a mutation in the proximal CACC has a nearly similar effect as the loss of the entire 5′\(\beta\) promoter. It may be that loss of (or decreased) binding of a specific protein or a complex of proteins to this segment of DNA results in a decrease in or absence of \(\beta\)mRNA formation and, indirectly, in an increase in 4mRNA formation because protein binding at the CACC box of the 5′\(\beta\) promoter is not affected. Such a difference would not exist when the \(\beta\)-thal mutation is outside this promoter region and the formation of \(\beta\)mRNA (although ineffective) is not influenced by this mutation. Another mechanism explaining the high \(\delta\) chain production is that the deletion of the \(\beta\)-globin gene promoter or the mutations in the TATA (+29, A → G) or CACC (−88, C → T) boxes

\[\text{Probes}\\
\text{Normal:} \quad 5'-\text{CTTTGGGA} - \text{TCTGTCCACT}-3'\\
\text{Mutant:} \quad 5'-\text{CTTTGGGA}[A]\text{TCTGTCCAC} -3'\\
\]
partially removes competition for limiting transcription factors, which makes these more readily available to the δ-globin gene promoter. However, this would affect equally the promoters of both β-globin genes and not only the δ-globin gene in cis to the β-thal allele.

REFERENCES

11. Diaz-Chico JC, Yang KG, Kutlar A, Reese AL, Aksoy M, Huisman THJ: An ~300 bp deletion involving part of the 5' β-globin gene region is observed in members of a Turkish family with β-thalassemia. Blood 70:583, 1987

ACKNOWLEDGMENT

The authors are indebted to Dr K. Punt (Utrecht, The Netherlands) and Dr F. Bueno de Mesquita, Dr J. Schaad, and to T. Merceur (Paramaribo, Surinam) for their help in obtaining some of the blood samples.
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