Globin Chain Synthesis in HbD (Punjab)-β-Thalassemia

By Ronald F. Rieder

A 23-yr-old man of Greek-Italian ancestry with mild anemia was found to be heterozygous for HbD (Punjab) β121 glu → gln and β-thalassemia. HbA was not detected upon electrophoresis of the subject’s hemolysate, and no synthesis of βA globin was demonstrated after incubation of peripheral blood or bone marrow with 3H-leucine. The thalassemia gene was thus of the β variety. The βD/α synthesis ratios were almost equally unbalanced in the blood and bone marrow: 0.53 and 0.61, respectively. The mother of the propositus had β-thalassemia trait. In peripheral blood the βA/α synthesis ratio was 0.38. The mutant βD gene thus appeared potentially capable of directing the synthesis of globin chains as efficiently as a normal βA gene. The mildness of the HbD-β-thalassemia syndrome appeared to be due to the maintenance of a relatively high total β/α synthesis ratio in the presence of a physiologically neutral structural mutation.

HbD (PUNJAB), β121 glu → gln, was the third abnormal human hemoglobin to be discovered. Originally termed HbD Los Angeles, it was first described in a family of mixed English, Irish, and American Indian ancestry. HbD (Punjab) has subsequently been well documented in several different widely separated ethnic groups, although initially there was some confusion with other electrophoretically similar but chemically distinct hemoglobins.

In the Sikhs of the Punjab region of India, HbD has an incidence of 2%-3%. In other races it occurs sporadically at a much lower frequency. The present paper describes in vitro studies of hemoglobin synthesis in peripheral blood and in bone marrow from an individual with chemically documented HbD (Punjab)-β-thalassemia.

MATERIALS AND METHODS

Clinical Hematology

Hematologic studies were performed according to standard methods. Hemoglobin electrophoresis on starch gel was done at pH 8.6 using a Tris-EDTA-borate buffer. HbA2 was measured by photometric scanning of cellulose acetate strips after hemoglobin electrophoresis (Helena Laboratories, Beaumont, Tex.). HbF was determined by the technique of Betke et al.

Fingerprint Analysis

Globin was prepared from membrane-free hemolysates using the acid-acetone method. βD (Punjab) was purified by column chromatography on carboxymethyl cellulose in 8 M urea followed by aminoethylation and trypsin digestion. High-voltage paper electrophoresis of the
tryptic digests was done using a pyridine-acetic acid-water buffer at pH 4.7. Butanol-acetic acid-pyridine-water, 15:3:10:12 was the solvent for the descending chromatographic step. Automatic amino acid analysis was done on material eluted from the fingerprints and hydrolyzed in 6 N HCl.

Hemoglobin Synthesis in Vitro

Washed peripheral blood erythrocytes and bone marrow cells were incubated in the medium of Lingrel and Borsook for 30 min in the presence of 3H-leucine. The entire hemolysate including membranes was converted to globin and the constituent polypeptide chains separated by column chromatography. The column eluate was monitored by liquid scintillation counting of 1-ml aliquots from each fraction. Relative total incorporation of radioactivity into individual chains was measured by summing the radioactivity in the 1-ml samples or by pooling all the fractions in each peak and counting a 1-ml aliquot.

RESULTS

Clinical

R.P., a 23-yr-old man, had been told 4 yr previously that he had thalassemia minor. At that time an enlarged spleen was noted. Prior medical history was otherwise negative. During a recent hospitalization for a respiratory infection, an abnormal hemoglobin was discovered by electrophoresis, and the level of HbA2 was reported to be elevated. The patient’s mother, M.B., who was of Italian origin, was reported to have a normal hemoglobin electrophoretic pattern with an elevated amount of HbA2. The patient’s father, who was of Greek origin, was unavailable for study.

Upon physical examination the patient was found to be a well-developed athletic man. The only abnormality was a splenic tip palpable just below the costal margin.

Table 1 presents the clinical hematologic data of the patient. There was slight anemia with a hematocrit value of 38.5. The red cell indices were microcytic with a mean corpuscular hemoglobin of 24.3 pg and a mean corpuscular volume of 78.6 cu μ. The reticulocyte count was elevated to 4.2%. The peripheral blood film showed moderate anisocytosis and poikilocytosis and frequent target cells.

Starch-gel electrophoresis at pH 8.6 revealed a single major hemoglobin band cathodic to HbA in the position of HbS or D (Fig. 1). A sickle preparation was negative, and upon agar gel electrophoresis at pH 6.1 the major hemoglobin band was found in the position of HbA or D. HbA2 was elevated to 6.4% (normal, 2% - 4%) but the fetal hemoglobin level was normal (normal < 1.5%) (Table 1).

The patient’s mother had a minor degree of hypochromia and microcytosis (Table 1). Her peripheral blood film showed slight anisocytosis and poikilocytosis with occasional target cells. Starch-gel electrophoresis revealed only HbA and HbA2. The level of HbA2 was elevated to 6.3%, and the percentage of fetal hemoglobin was 0.85%.

<table>
<thead>
<tr>
<th>Table 1. Clinical Hematologic Data</th>
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<tbody>
<tr>
<td>Subject</td>
</tr>
<tr>
<td>R.P. (son)</td>
</tr>
<tr>
<td>M.B. (mother)</td>
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Fig. 1. Starch-gel electrophoresis at pH 8.6 of normal (upper), HbD-thalassemia (middle), and sickle trait (lower) hemolysates. HbA is absent from the D-thalassemia specimen, indicating that the thalassemia gene is of the $\alpha^-$ type. HbD (Punjab) which migrates like HbS in this system is found in the position of HbA on agar-gel electrophoresis at pH 6.1 (not shown). Benzidine stain. The anode is toward the right.

**Fingerprint and Amino Acid Analysis**

The $\alpha$- and $\beta$-chains of the abnormal hemoglobin were separated by carboxymethyl cellulose chromatography. The $\beta$-chain, which eluted in a position between that expected for $\beta^\alpha$ and $\alpha$ globin, was fingerprinted. $\beta^\alpha T_{p}X_{III}$ was absent from its customary position and was replaced by a more positively charged peptide. After acid hydrolysis, amino acid analysis of the new peptide...
Table 2. Globin Chain Synthesis

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sample</th>
<th>Total Radioactivity (cpm)</th>
<th>$\beta^A$</th>
<th>$\beta^D$</th>
<th>$\alpha$</th>
<th>$\beta/\alpha$</th>
</tr>
</thead>
<tbody>
<tr>
<td>R.P.</td>
<td>Peripheral blood</td>
<td>—</td>
<td>31,998</td>
<td>60,053</td>
<td>0.53</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bone marrow</td>
<td>—</td>
<td>264,288</td>
<td>413,924</td>
<td>0.61</td>
<td></td>
</tr>
<tr>
<td>M.B.</td>
<td>Peripheral blood</td>
<td>26,030</td>
<td>—</td>
<td>67,805</td>
<td>0.38</td>
<td></td>
</tr>
</tbody>
</table>

revealed a composition identical to that expected for $\beta^A$Hb XIII. Since acid hydrolysis converts glutamine to glutamic acid, the abnormal hemoglobin was identified as HbD (Punjab) $\beta^{121}$ glutamic acid $\rightarrow$ glutamine.

Globin Chain Synthesis

Peripheral blood and bone marrow from the propositus, R.P., were incubated for 30 min in the presence of $^3$H-leucine. Figure 2 shows the column chromatography of the globin prepared from the peripheral blood specimen. Radioactive globin peaks were found only in the positions of $\beta^D$ and $\alpha$. The $\beta^D/\alpha$ synthesis ratio was 0.53 (Table 2). The $\beta^D/\alpha$ synthesis ratio in the bone marrow specimen was similarly unbalanced (Table 2, Fig. 3).

Globin chain synthesis was also studied in the peripheral blood of the subject's mother, M.B. There was unbalanced chain synthesis; the $\beta^A/\alpha$ ratio was 0.38 (Table 2, Fig. 4).

DISCUSSION

R.P., the propositus in this study, is clearly heterozygous for HbD (Punjab) and $\beta$-thalassemia. The clinical hematologic data including the microcytic, hypochromic red cell indices and the elevated level of HbA$_2$ indicate $\beta$-thalassemia trait. The mother, M.B., also demonstrates an increased proportion of HbA$_2$ and is a carrier of the gene for $\beta$-thalassemia. The father who could not be studied undoubtedly is a carrier of HbD.

The gene for thalassemia in this family is of the $\beta^+$ variety and results in complete suppression of the synthesis of $\beta^A$ globin. This conclusion is indicated by the complete absence of HbA in the hemolysate of the propositus and by the failure of his reticulocytes and bone marrow to demonstrate synthesis of $\beta^A$ when incubated in vitro with radioactive amino acid. The absence of one functional gene for $\beta$ globin results in the imbalance in $\alpha$ and $\beta$ synthesis with $\beta$-chain deficit that is demonstrated in vitro in the erythroid cells of both the son and the mother.

The synthetic imbalance in vitro appeared to be somewhat greater in the mother than in the son. Parent-child differences in $\beta/\alpha$ synthesis ratios of this degree in peripheral blood have been previously reported in subjects with $\beta$-thalassemia trait. The magnitude of the imbalance in globin production noted in the present subjects has also been seen previously in mild $\beta$-thalassemia trait and in Hb Lepore trait. The degree of anemia and decreased hemoglobinization of erythrocytes were fairly comparable in R.P. ($\beta^D = 11.4$ pg) and M.B. ($\beta^A = 13.0$ pg), indicating that there was an equivalent capacity for compensatory $\beta$ globin synthesis directed by the functioning $\beta^A$ and $\beta^D$ genes. This finding is of interest since subjects with HbD trait synthesize more HbA
than HbD. The proportion of the mutant hemoglobin in heterozygotes is generally 35%–45% \(^{20,21}\) although individuals have been reported with as little as 31% \(^{22}\) and as much as 50% HbD. \(^5\) Similar compensatory synthesis of \(\beta^S\) in sickle thalassemia is also seen. \(^7\)

The imbalance in \(\beta\)- and \(\alpha\)-chain production was approximately the same in the marrow and peripheral blood of R.P. This result contrasted with the findings of Schwartz and Gill in patients with HbS-\(\beta\)-thalassemia \(^7\) and \(\beta\)-thalassemia trait. \(^8\) Those investigators reported that, while in reticulocytes there was a deficit in total \(\beta\) globin synthesis relative to \(\alpha\) globin, in marrow specimens there was equal incorporation of radioactivity into the two types of
chains. Other observers have also indicated that total non-α/α globin synthesis was balanced in β-thalassemia heterozygotes. The significance of those prior observations and the reasons why they differ from the present study are not clear. The present experiments were done on globin prepared from whole lysates including the membrane fraction. Some of the previous studies were done on hemolsates from which the red cell stroma had been removed prior to the acid-acetone precipitation step. It is possible that a portion of the unstable excess α-chains were removed with the stromal fraction. In addition, the present incubations were done for only 1/2 hr, while other investigators have generally incubated the marrow specimens for 1–2 hr. It is known that with prolonged incubation a proportion of the excess radioactive α-chains becomes associated with a stromal pool.

Not all studies have invariably shown balanced globin synthesis in the marrow of thalassemia heterozygotes. Clegg and Weatherall reported a marked degree of synthetic imbalance in the marrows of three subjects with thalassemia trait that was equal to that seen in the present study. Those workers presented evidence that a radioactive contaminant might cochromatograph with the β globin to raise spuriously the apparent β/α synthesis ratio. Wood and Stamatoyannopoulos found evidence suggesting unbalanced globin synthesis in red cell precursors at all stages of development in marrow from two thalassemia heterozygotes and one patient with sickle thalassemia. Another subject with sickle thalassemia and a total non-α/α synthesis ratio of 0.59 has also been reported. Such imbalance in globin synthesis implies that there is a large pool of free α-chains in the marrow of thalassemia heterozygotes; this has been demonstrated. The free α-chains are apparently efficiently degraded since α-chain inclusions are not seen.

There was no significant anemia or morbidity in the present patient with HbD-β-thalassemia. The degree of morbidity associated with this syndrome seems to be quite variable, but only a few probable examples of simultaneous heterozygosity for HbD (Punjab) and β-thalassemia have appeared in the literature. No prior study of globin synthesis in affected subjects has been published. In all reports, except one, the thalassemia gene involved seems to have been of the β⁰ variety as judged by the absence of HbA upon electrophoresis.

Hynes and Lehmann reported a Persian girl with slight anemia who exhibited only a HbD (presumably D Punjab) on electrophoresis. The coexistence of thalassemia was suspected because of low red cell indices and a characteristic thalassemic blood film, but the father was hematologically normal and the mother was not studied. Sukumaran, Sanghvi, and Nazreth reported four individuals in two families from India who exhibited only a HbD (presumably D Punjab) upon electrophoresis and in whom genetic evidence supported the coexistence of a gene for thalassemia and eliminated the possibility of heterozygosity for β⁰. Two of the subjects were not anemic. Schneider and associates encountered a woman of English, Scotch, and Irish ancestry with a microcytic, hypochromic anemia having a hematocrit value of 28%. Hemoglobin electrophoresis and chromatography revealed 82.7% HbD (Punjab), 8% HbA, 5.3% HbA₂, and 5% HbF, indicating heterozygosity for the structural ab-
normality and a $\beta^+\text{-thalassemia.}$ An Indian child with thalassemic-like red cells, 21% HbF, 79% HbD (presumably D Punjab), and severe anemia (hematocrit value 23%) was reported by Jain, Andleigh, and Mehta. The child's father, who was not anemic, appeared to be homozygous for HbD while the mother exhibited evidence of $\beta$-thalassemia trait. Well-documented HbD (Punjab)-$\beta$-thalassemia was reported in a boy of Bulgarian-Jewish ancestry with hemolytic anemia of moderate severity and 91% HbD, the rest being HbA2 and HbF. An Italian woman has also been described with 96.5% HbD (not chemically defined), 3.5% HbA2, and a hematocrit of 32%. The father was a carrier of a HbD, while the mother appeared to have thalassemia minor. Different degrees of $\alpha/\beta$ synthesis imbalance might have been responsible for the varying clinical severity in these cases, although other nonhematologic factors cannot be discounted.

Despite some possible instances of moderate morbidity, HbD (Punjab)-$\beta^+$-thalassemia seems to be a milder syndrome than HbE-$\beta^+$-thalassemia, another hemoglobinopathy prevalent in Southeast Asia. The latter disorder is characterized by a moderate to severe anemia resembling thalassemia major. A $\beta^E/\alpha$ synthesis ratio quite similar to the $\beta^D/\alpha$ ratio in the present study has recently been reported in a severely anemic girl with HbE-$\beta^+\text{-thalassemia.}$ One other report indicates somewhat greater imbalance in that disorder. Instability of HbE may account for the difference in the degree of anemia observed when a $\beta^+\text{-thalassemia}$ gene is in combination with a $\beta^D$ or $\beta^E$ gene.

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