Homozygous State for Hb Constant Spring
(Slow-moving Hb X Components)

By Lie-Injo Luan Eng (Luan Eng Lie-Injo), J. Ganesan, J.B. Clegg, and D.J. Weatherall

A 12-yr-old Malay boy who was studied because his youngest brother and both his parents had the slow-moving Hb X components (earlier reported to lead to Hb H disease when combined with α-thalassemia) was found to be homozygous for the same slow-moving components. He had splenomegaly and a just palpable liver, mild anemia with microcytosis, hypochromia, slight morphologic changes of the red blood cells, and slight reticulocytosis. Of eight children in the family, six had the trait for the abnormality, one was normal, and one, the propositus, was homozygous. Structural studies of the isolated abnormal hemoglobin showed it to be identical to Hb Constant Spring (Hb CoSp), an α-chain variant with 172 residues instead of the usual 141, the additional 31 being attached to the C-terminal end. In addition to the abnormal α variant for which the propositus was homozygous, he also had normal Hb A and normal Hb A2 with normal α-chains. If the theory that Hb CoSp is due to a structural mutation affecting the terminator codon is correct, this case provides evidence for a duplication of the gene for α-chain production. Results of study of several erythrocyte enzymes are also reported.

Hemoglobin H (Hb H) disease in Malaysia assumes two forms, one of which is associated with slow-moving hemoglobin components designated Hb X. In those subjects with these slow-moving hemoglobin components, they were invariably found in one but not both of the parents.1-5 Slowly migrating hemoglobin components were reported earlier in patients with Hb H disease in Greece as Hb Athens,3 and in Thailand as Hb Thai4; their significance and mode of inheritance were, however, not demonstrated. Similar slow-moving components were reported in a Chinese family from Jamaica with Hb H disease and designated Hb Constant Spring (Hb CoSp), a variant which was shown to have abnormal α-chains with 31 additional amino acids at the C-terminal end.5,6 The abnormal slow-moving hemoglobin was also reported in a Chinese family with Hb H disease in the United States,7 and in Hong Kong.8 Subsequent structural studies on the slow-moving hemoglobin components from Greece, Thailand, Malaysia, and Hong Kong showed them to be identical to Hb Constant Spring from Jamaica.9

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### Table 1. Hematologic Findings in a Patient Heterozygous for Hb CoSp and in His Family

<table>
<thead>
<tr>
<th>Subject</th>
<th>Sex and Age</th>
<th>Hb (g/100 ml)</th>
<th>RBC (mil/ cu mm)</th>
<th>PCV (%)</th>
<th>MCV (cu μ)</th>
<th>MCH (μg)</th>
<th>MCHC (%)</th>
<th>Ret (%)</th>
<th>Hb Pattern</th>
<th>Serum iron (μg/100 ml)</th>
<th>Hb CoSp (%)</th>
<th>Hb A2 (%)</th>
<th>Hb F (%)</th>
<th>Hb Bart’s (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propositus</td>
<td>M 12</td>
<td>11.3</td>
<td>4.82</td>
<td>36.5</td>
<td>75.7</td>
<td>23.4</td>
<td>31.0</td>
<td>7.6</td>
<td>A + A2 + CoSp</td>
<td>71</td>
<td>5.2</td>
<td>1.8</td>
<td>13</td>
<td>Not detectable</td>
</tr>
<tr>
<td></td>
<td>after iron th.</td>
<td>10.3</td>
<td>4.89</td>
<td>34.5</td>
<td>70.6</td>
<td>21.1</td>
<td>29.9</td>
<td>5.7</td>
<td>A + A2 + CoSp</td>
<td>89</td>
<td>5.0</td>
<td>2.0</td>
<td>20</td>
<td>Trace</td>
</tr>
<tr>
<td>Father</td>
<td>M 32</td>
<td>13.4</td>
<td>5.52</td>
<td>41.5</td>
<td>75.2</td>
<td>24.3</td>
<td>32.3</td>
<td>1.6</td>
<td>A + A2 + CoSp</td>
<td>165</td>
<td>0.5</td>
<td>3.1</td>
<td>14</td>
<td>Not detectable</td>
</tr>
<tr>
<td>Mother</td>
<td>F 29</td>
<td>12.4</td>
<td>4.58</td>
<td>40.0</td>
<td>87.3</td>
<td>27.0</td>
<td>31.0</td>
<td>1.2</td>
<td>A + A2 + CoSp</td>
<td>69</td>
<td>1.2</td>
<td>3.0</td>
<td>15</td>
<td>Not detectable</td>
</tr>
<tr>
<td>Sister 1</td>
<td>F 10</td>
<td>12.7</td>
<td>5.16</td>
<td>41.0</td>
<td>79.5</td>
<td>24.6</td>
<td>30.1</td>
<td>0.8</td>
<td>A + A2 + CoSp</td>
<td>110</td>
<td>1.0</td>
<td>3.1</td>
<td>10</td>
<td>Not detectable</td>
</tr>
<tr>
<td>Sister 2</td>
<td>F 6</td>
<td>12.2</td>
<td>5.16</td>
<td>39.5</td>
<td>76.6</td>
<td>23.6</td>
<td>30.9</td>
<td>2.2</td>
<td>A + A2 + CoSp</td>
<td>133</td>
<td>0.6</td>
<td>2.5</td>
<td>12</td>
<td>Not detectable</td>
</tr>
<tr>
<td>Brother 1</td>
<td>M 13</td>
<td>13.4</td>
<td>5.21</td>
<td>42.0</td>
<td>80.6</td>
<td>25.7</td>
<td>31.9</td>
<td>1.8</td>
<td>A + A2</td>
<td>—</td>
<td>—</td>
<td>3.0</td>
<td>—</td>
<td>Not detectable</td>
</tr>
<tr>
<td>Brother 2</td>
<td>M 7</td>
<td>12.2</td>
<td>4.77</td>
<td>40.5</td>
<td>84.9</td>
<td>25.6</td>
<td>30.1</td>
<td>0.6</td>
<td>A + A2 + CoSp</td>
<td>—</td>
<td>0.5</td>
<td>2.8</td>
<td>14</td>
<td>Not detectable</td>
</tr>
<tr>
<td>Brother 3</td>
<td>M 4</td>
<td>10.0</td>
<td>5.81</td>
<td>35.5</td>
<td>61.1</td>
<td>17.2</td>
<td>28.2</td>
<td>0.8</td>
<td>A + A2 + CoSp</td>
<td>30</td>
<td>0.6</td>
<td>2.8</td>
<td>12</td>
<td>Not detectable</td>
</tr>
<tr>
<td>Brother 4</td>
<td>M 2</td>
<td>12.9</td>
<td>6.26</td>
<td>42.0</td>
<td>67.1</td>
<td>20.6</td>
<td>30.7</td>
<td>2.4</td>
<td>A + A2 + CoSp</td>
<td>59</td>
<td>1.1</td>
<td>2.4</td>
<td>18</td>
<td>Not detectable</td>
</tr>
<tr>
<td>Brother 5</td>
<td>M (Cord blood)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>A + A2 + CoSp + Bart's</td>
<td>—</td>
<td>1.3</td>
<td>—</td>
<td>—</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>M 8 days</td>
<td>16.8</td>
<td>5.38</td>
<td>50.0</td>
<td>92.9</td>
<td>31.2</td>
<td>33.6</td>
<td>1.8</td>
<td>A + A2 + CoSp + Bart's</td>
<td>—</td>
<td>0.8</td>
<td>58.3</td>
<td>2.5</td>
<td></td>
</tr>
<tr>
<td>Aunt</td>
<td>(Mother’s side)</td>
<td>14.0</td>
<td>4.64</td>
<td>42.5</td>
<td>91.6</td>
<td>30.1</td>
<td>32.9</td>
<td>0.6</td>
<td>A + A2</td>
<td>133</td>
<td>—</td>
<td>3.6</td>
<td>—</td>
<td>Not detectable</td>
</tr>
<tr>
<td>(Father’s side)</td>
<td>F 57</td>
<td>11.7</td>
<td>4.71</td>
<td>38.0</td>
<td>83.8</td>
<td>24.8</td>
<td>30.1</td>
<td>0.6</td>
<td>A + A2 + CoSp</td>
<td>120</td>
<td>0.5</td>
<td>2.5</td>
<td>1.7</td>
<td>Not detectable</td>
</tr>
</tbody>
</table>

* Age in yr, unless otherwise noted

1 Values taken as the normal range in the Department of Biochemistry: Male 80–175, female 60–160.
Hb CoSp in Malaysia, designated slow-moving Hb X components before they were shown to be identical to Hb CoSp, occurred in Kuala Lumpur, Malaysia in 2.24% of Malays, 0.66% of Chinese, and 0.16% of Indians. Higher frequencies were found in certain groups of Malayan aborigines.

The homozygous condition for these slow-moving Hb X components found in a Malay boy was briefly reported by Lie-Injo. In this paper we describe in detail the clinical and hematologic findings and the hemoglobin analysis in that homozygous Malay boy. The results of studies of several erythrocyte enzymes are also reported.

MATERIALS AND METHODS

Hematologic examinations were carried out according to standard methods. Electrophoresis of hemoglobin was done on starch gel using Tris-EDTA boric acid buffer at pH 8.6 and 8.0, in discontinuous Tris-boric acid buffer at pH 9.5, and phosphate buffer at pH 7.7. Cellulose acetate electrophoresis was done in Tris-EDTA boric acid buffer at pH 8.9, and agar gel electrophoresis in citrate buffer at pH 6.2. Hemoglobin components were quantitated by the cellulose acetate electrophoretic method of Marengo-Rowe. Alkali-resistant hemoglobin was estimated by the method of Singer et al. Isolation and structural studies of the abnormal hemoglobin were carried out as described by Clegg et al.

Glucose-6-phosphate dehydrogenase (G6PD) activity was estimated by the method of Zinkham et al. as recommended by the World Health Organization, glutathione level by the method of Beutler et al., glutathione reductase (GR) activity by the method of Beutler, glutathione peroxidase (GP) activity by the method of Paglia and Valentine, pyruvate kinase (PK) activity by the method of Valentine and Tanaka, and methemoglobin reductase activity by the method of Hegesh et al.

CASE REPORT

A 12-yr-old Malay boy was studied because his youngest brother was found to have slow-moving Hb X components and Hb Bart’s at birth while both his parents had the same slow-moving Hb X components.

The boy was well-nourished, slightly small for his age, and was not jaundiced. His spleen was slightly enlarged and reached two finger breadths below the costal margin while his liver was just palpable. No other physical abnormalities were found. He was examined twice, the second time after the completion of a course of iron therapy, given because he was found to have mild anemia and microcytosis when he was first examined.

Hematological findings on both occasions are listed in Table 1. The hemoglobin level was slightly reduced at 10–11 g/100 ml, and the MCH was markedly reduced. There was a slight reticulocytosis. WBC and platelet counts were normal. The peripheral blood smear showed slight anisocytosis and poikilocytosis of the red blood cells (Fig. 1). A few target cells were seen and, in addition, there were many peculiar small irregularly shaped cells. No normoblasts could be detected. Staining with methyl violet did not reveal Heinz bodies, nor could any Hb H inclusion bodies be demonstrated. A sickling test of the erythrocytes was negative. The erythrocytes showed a slightly decreased osmotic fragility; hemolysis started at 0.36% and was complete at 0.20% (control at 0.40% and 0.32%, respectively). Serum iron, examined about 2 wk after completion of an iron course, was 89 μg/100 ml (normal) and serum bilirubin was 1.1 mg/100 ml total, 0.6 mg/100 ml direct reacting, and 0.5 mg/100 ml indirect reacting. The direct Coomb’s test was negative. Haptoglobin could not be demonstrated on starch gel electrophoresis. G6PD activity, examined by the dye decoloration test of Motulsky and Campbell-Kraut and by the method of Zinkham et al., was found to be elevated. Urobilin and urobilinogen were increased in the urine, but hemoglobin, bilirubin, and hemosiderin were not detected. The level of glutathione and activities of glutathione peroxidase, glutathione reductase, methemoglobin reductase, and pyruvate kinase are listed in Table 2.

Family Study

Both parents were Malay. They had eight children including the propositus and his newborn brother. All the relatives were healthy and without physical abnormalities; their hematologic findings are listed in Table 1. The father, sister No. 2, and brothers Nos. 3 and 4 had microcytosis of the red blood cells.
and slight aniso- and poikilocytosis in the peripheral blood smears with a few oval cells and irregularly shaped cells. The mother, grandmother, and sister No. 1 had similar slight changes of the red blood cells, although they had normal MCV. The others did not show significant changes. No Hb H inclusion bodies were seen, and the reticulocyte, WBC, and platelet counts were normal. Serum bilirubin levels were within normal limits. Serum iron levels are listed in Table I. G6PD activity was normal in all family members.

**Hemoglobin Studies**

On starch gel electrophoresis a large amount of Hb A, a small amount of Hb A2, and several minor hemoglobin components, moving more slowly than Hb A2, were found. Four of these slow-moving components were clearly seen in the propotitus after staining with benzidine (Fig. 2). They were much more pronounced than those seen in the parents and other siblings with the same abnormality, who usually showed only two of the slow-moving components instead of four. Sometimes another hemoglobin component moving slightly faster than Hb A2 was demonstrable (Fig. 3). This last component was not always clear, and its appearance was not always due to the hemolysate being old because it could be seen when the freshly prepared hemolysate was run on the same day on which the blood was drawn. When examined for the second time, the patient had a trace of a hemoglobin component with the mobility of Hb Bart's not seen at the first examination on starch gel electrophoresis in Tris-EDTA boric acid buffer at pH 8.6 and in phosphate buffer at pH 7.7. No Hb H could be demonstrated. Quantitative analysis of hemoglobin components by cellulose acetate electrophoresis gave the following results on the first occasion: Hb A 93.0%, Hb A2 1.8%, combined slow-moving hemoglobin components 5.2%. On the second occasion, similar results were obtained (Table I). In the parents and siblings who carried the ab-
normal X components, quantitative analysis showed levels of the abnormal hemoglobin to range between 0.5% and 1.3%. Alkali-resistant hemoglobin in the patient was found to be 1.3% on the first examination and 2% on the second examination. The level of alkali-resistant hemoglobin was within normal limits in all relatives examined (Table 1).

Four of the slow-moving components were isolated and purified by chromatography on Amberlite IRC-50.* Structural studies on the abnormal α-chains were carried out according to methods described earlier." These showed that two of the components were identical to the Hb CoSp1 and Hb CoSp2 frac-
tions of Hb Constant Spring, which has abnormal \( \alpha \)-chains with 172 residues instead of the usual 141 due to an additional 31 residues being attached to the C-terminal end of the \( \alpha \)-chains. A third component, which corresponds to a component earlier designated CS3, but designated CoSp5 in Fig. 3, was shown to have \( \alpha \)-chains which extended to the Trp residue 154 of the \( \alpha \)-CoSp chain. The fourth minor component corresponding to CoSp3 in Fig. 2 was not studied further. Also, the components with the mobility of Hb A and Hb A1 were isolated and analyzed. The hemoglobin with the mobility of Hb A had normal \( \alpha \)- and normal \( \beta \)-chains, while the component with the mobility of Hb A1 had normal \( \alpha \)- and normal \( \beta \)-chains. There were thus no abnormal globin chains in the blood of the patients other than those in the abnormally slow-moving hemoglobin components.

DISCUSSION

From the clinical, hematologic, and biochemical findings and the family studies, it is clear that the patient described in this paper must be homozygous for Hb CoSp. Although he was discovered only because of a routine family study, he had definite clinical and hematologic abnormalities. He had splenomegaly and hypochromic microcytic anemia even after iron therapy. The hematologic findings, although mild, were clearly abnormal, with a reticulocytosis and other changes in the peripheral blood, and the biochemical findings were quite different from those in the trait carriers of Hb CoSp. Several of the other members of the family who were trait carriers of Hb CoSp also had some microcytosis, hypochromia, and slight morphological changes of the red blood cells (Table 1). The microcytosis in the father and sister No. 2 was accompanied by normal serum iron levels while the serum iron in brothers No. 3 and 4 was low. The microcytosis in the father and sister No. 2 are therefore probably due to the presence of abnormal Hb CoSp. That the microcytosis and hypochromia in brothers No. 3 and 4 who had low serum iron levels were due or partly due to iron deficiency cannot be excluded. In a separate study of many carriers of Hb CoSp, we have found that the abnormal hemoglobin can be accompanied by slight microcytosis and morphologic changes of the erythrocytes although more often it is not (Lopez, Lie-Injo, and Lopes, unpublished).

The amount of abnormal hemoglobin in the patient was five to ten times that found in the parents, who were both trait carriers of Hb CoSp. Also, four abnormal components were easily demonstrable. Clegg et al. suggested that Hb CoSp is derived from Hb CoSp1 by proteolysis, and it is likely that the other abnormal components are produced in the same way. The patient’s level of alkali-resistant hemoglobin was sometimes at the borderline between normal and abnormal, and a trace of Hb Bart’s was sometimes detectable.

Contrary to the usual finding in homozygous cases for an abnormal hemoglobin, only about 5% of the patient’s total amount of hemoglobin was of abnormal type, while a large amount of normal Hb A was found. Usually, in a person homozygous for a \( \beta \) variant of hemoglobin most or all the hemoglobin found is of the abnormal type with some Hb F often present, but no Hb A. This is also true for the homozygous state of one known \( \alpha \)-chain variant, the Hb J Tongariki. However, the propositor, who is obviously homozygous for the abnormal Hb CoSp with abnormal \( \alpha \)-chains, also has at the same time normal \( \alpha \)-chains in Hb A and Hb A1. If Hb CoSp is due to a structural mutation affecting the gene-terminating codon, which at present is the most likely explanation, the propositor must have two pairs of genes controlling the structure of \( \alpha \)-chains, one normal and one abnormal. This would not be the case, however, if Hb CoSp were due to the presence of a suppressor tRNA mutant, which was suggested as an unlikely alternative explanation.
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for Hb CoSp. The suggestion of Lehmann and Carrell that the α-chain gene may be duplicated has already found evidence in the reports of Brimhall et al. and Hollan et al., who described two different abnormal types of α-chain variants of hemoglobin in individuals possessing normal Hb A as well. Assuming that the theory of structural mutation is correct, the patient described in the present paper presents further direct evidence for the existence of two α-chain loci in Malays. Further, a certain type of Hb H disease is thought to be due to the combination of α-thalassemia with Hb CoSp (Hb X components). If the α-thalassemia is of the severe type, it is expected that in a combination with an α-chain variant the production of Hb A is completely suppressed, and normal α-chains are not found. Since the Hb H disease associated with Hb CoSp, Hb A is present in large amounts one may also conclude from this that the α-chain gene is duplicated. However, in Hb Q-α-thalassemia (Hb Q-H disease) no Hb A is found, and one has to conclude that there is only one locus for α-chain in such cases. Also, the absence of Hb A in cases homozygous for Hb J Tongariki, an α-chain variant found in Melanesians, leads to the conclusion that the Melanesians have only one α-chain locus. Since both Hb H disease with Hb CoSp and Hb Q-α-thalassemia occur in Chinese it seems possible that both single and duplicated α-chain loci occur in this population, provided that the structural mutation theory for Hb CoSp is correct.

The newborn brother of the patient had Hb CoSp in the blood. As reported by Lie-Injo Hb CoSp (slow-moving Hb X components) in the newborn is usually accompanied by Hb Bart’s. In Malaysia Hb Bart’s in the newborn does not always mean that the newborn has α-thalassemia, because Hb Bart’s can accompany different types of abnormalities involving α-, β-, and γ-chain production, which have nothing to do with α-thalassemia. Erythrocyte enzyme studies showed the patient’s glutathione level to be low and the activities of G6PD, 6PGD, PK, and GP to be very much elevated. Also, the GR activity was at the higher limits of normal for our findings in Malaysians. Diaphorase activity was within normal limits. The increased activity of the glycolytic enzymes in the patient was probably due to a younger erythrocyte population. Such an increase was not found in the parents, who were trait carriers of Hb CoSp. The low glutathione level and high GR activity in the patient may have been due to increased utilization of reduced glutathione. Similar changes in glutathione level and erythrocyte enzyme activities were reported in patients with unstable Hb Kūln.

As far as we are aware the patient described in this paper is the first case of homozygous Hb CoSp so far reported. In the Temuan group of Malayan aborigines Hb CoSp occurs with a frequency of 3.2%, so that it is to be expected that the homozygous condition is not very rare. A systematic search for such homozygous cases among the Temuan has indeed led to the finding of another family with three members homozygous for Hb CoSp.

ACKNOWLEDGMENT

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