Phenotype-genotype correlation in haemoglobin H disease in childhood

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SUMMARY In this study we used restriction endonuclease mapping to characterise the molecular defect responsible for haemoglobin H disease in 14 Sardinian children. The resulting genotypes were then correlated with the respective clinical and haematological phenotypes. We found that patients with the combination of non-deletion \(\alpha^+\)-thalassaemia \([(\alpha_\alpha)^{th}]\) and deletion \(\alpha^+\)-thalassaemia \((--\text{Med})\) have a more severe phenotype than that resulting from the interaction of deletion \(\alpha^-\)thalassaemia \((-\text{Med})\) and \(\alpha^+\)-thalassaemia \((-\alpha)\) determinants. Clinically, presentation was earlier and with moderate anaemia or haemolytic crisis, enlargement of the liver and spleen, and thalassaemic bone changes. Haematologically, the anaemia was more severe and there were higher bilirubin levels, reticulocyte counts, Hb H levels, and percentage of red blood cells with inclusion bodies. These results suggest that in those Hb H disease patients with the non-deletion \([(\alpha_\alpha)^{th}]\) determinant, two \(\alpha\) globin genes produce fewer \(\alpha\) globin chains than a single \(\alpha\) globin locus.

The two \(\alpha\) globin structural genes lie on chromosome 16 linked to one \(\zeta\) globin gene, a \(\zeta\)-like gene \((\psi\zeta_1)\), and an \(\alpha\)-like pseudogene \((\psi\alpha_1)\) in the order \(5'\zeta_2\psi\zeta_1\alpha_1\alpha_2\alpha_3\). Several different lesions of this gene complex produce the \(\alpha\)-thalassaemias, a group of genetic disorders characterised by a reduced or absent output of \(\alpha\) globin chains within the red blood cells.

Haemoglobin H (Hb H) disease, the most important clinical form of \(\alpha\)-thalassaemia, is a hereditary microcytic anaemia most frequently found in South Asian and Mediterranean populations. Most commonly, this disorder results from the interaction of a deletion form of \(\alpha^-\)-thalassaemia \((-\alpha)\) with a deletion \(\alpha^+\)-thalassaemia \((-\alpha)\) determinant. However, this condition can also be produced either by the combination of a deletion form of \(\alpha^-\)-thalassaemia \((-\alpha)\) with a non-deletion \(\alpha^+\)-thalassaemia determinant \([(\alpha_\alpha)^{th}]\) or by the homozygous state of a non-deletion defect \([(\alpha_\alpha)^{th}]/(\alpha_\alpha)^{th}\), so far seen only in Saudi Arabs.

The \(\alpha^-\)-thalassaemia determinant, characterised by a complete suppression of \(\alpha\) globin chain production, includes haplotypes with loss of both \(\alpha\) globin structural loci as well as haplotypes with residual but non-functional \(\alpha\) globin genes.

The \(\alpha^+\)-thalassaemia determinant, characterised by a reduced output of \(\alpha\) globin chains, is most frequently caused by the deletion of one \(\alpha\) globin structural gene, but can also be produced by lesions that do not involve gross gene deletion (non-deletion \(\alpha^+\)-thalassaemia haplotypes). Such non-deletion gene lesions may result from point mutations in the termination codon of the \(\alpha_\alpha\) gene resulting in the production of elongated \(\alpha\) globin chains, short deletions such as the recently described five base pair deletion in the first intervening sequence of the \(\alpha_\alpha\) gene, single amino-acid substitution \((\alpha_\alpha)^{th}\), or other less well defined defects. Even at the phenotypic level, there is a remarkable variation in the clinical and haematological manifestations. Negroes with Hb H disease have a significantly milder clinical picture than Oriental or Mediterranean patients. However, significant phenotypic heterogeneity may be seen even within the same racial group.

Phenotypic diversity in genetic diseases may be the result of environmental or genetic causes or both. Environmental factors such as iron deficiency, infections, and drug administration have been seen to produce variations in the clinical severity of this disorder. There is, however, very little information on the relationship between the clinical phenotype and the molecular defect.

In this study, we compared the clinical phenotypes of Sardinian patients with Hb H disease resulting from different molecular defects. We found a more...
severe clinical and haematological picture in patients with a combination of deletion and non-deletion \(\alpha\)-thalassaemia \(-\alpha/-(\alpha\alpha)\) than in those with the more usual deletion defect \(-\alpha/-\).

Patients

We studied 14 Sardinian children, aged 1 to 14 years, with haemoglobin H disease. This diagnosis was established by means of haemoglobin electrophoresis at pH 8.6 and pH 7.0, brilliant cresyl blue staining of the peripheral blood, and globin chain synthesis analysis. None of the patients had Hb Constant Spring. Extensive clinical and haematological data from each of these patients were available as they had been regularly followed in our outpatient service. The molecular basis was investigated by restriction endonuclease mapping of DNA.

Methods

Haematological measurements were made with the Coulter Counter S standardised daily with a 4C commercial standard. The quantification of Hb H (\(\beta_4\)) and Hb Bart’s (\(\gamma_4\)) was performed in duplicate by elution of the bands after cellulose acetate electrophoresis using a phosphate system buffer pH 6.5. Haemoglobin A2 was quantified by DE-52 microchromatography and haemoglobin F by an alkali denaturation method. G6PD activity was determined according to WHO. Globin chain synthesis in peripheral blood reticulocytes was performed according to Kan et al.

DNA was prepared from white blood cells by phenol-chloroform extraction. Under conditions recommended by the manufacturers (Boehringer Mannheim, West Germany), 10 \(\mu\)g aliquots of DNA were digested with 20 to 30 units of restriction endonucleases \(Bgl\) II, \(Bam\) HI, and \(Eco\) RI for 24 hours. The digested DNA samples were fractionated on 0.8% agarose gels and transferred to nitrocellulose filters by Southern blotting. The filters were hybridised with a specific \(32P\)-labelled \(\alpha\) globin gene probe prepared by nick-translation, as described by Maniatis et al. from \(\alpha\) globin cDNA cloned in the plasmid JW 101. The filters were also hybridised with a \(\zeta\) globin specific probe prepared by nick-translation of a \(Hinf\) I fragment of the pBR\(\zeta\) plasmid.

Results

Restriction endonuclease analysis of DNA from nine of the 15 patients included in this study, either with \(Eco\) RI or \(Bam\) HI, showed only the single shortened fragment containing one \(\alpha\) locus \((-\alpha/-\)\)\(6,27,28\) (fig 1). Digestion of DNA from these subjects with \(Bgl\) II produced only a 16-kb \(\zeta\) globin specific fragment, which is typical of the rightward deletion type of unequal crossover leading to the formation of a single \(\zeta\) globin locus. DNA from all these patients with a single \(\zeta\) globin locus, digested with \(Bgl\) II and hybridised with a \(\zeta\) globin specific probe, yielded a 14-kb fragment, which is characteristic of the most common Mediterranean \(\alpha^+\)-thalassaemia haplotype, in addition to the 11-kb and 16-kb fragments, which are typical of the rightward deletion \(\alpha^+\)-thalassaemia haplotype (fig 2).

In the remaining five patients, \(Eco\) RI, \(Bam\) HI, and \(Bgl\) II Southern blots, hybridised with an \(\alpha\) globin specific probe, showed a normal arrangement of \(\alpha\) globin genes. Digestion of the DNA from these

![FIG 1] Restriction endonuclease map of the \(\alpha\)-like globin gene cluster (at the top) and sizes (in kb) of \(\alpha\) and \(\zeta\) specific fragments obtained after restriction endonuclease digestion of DNA from normal (\(\alpha\alpha\)) and \(\alpha\)-thalassaemia haplotypes. \(Bg = Bgl\) II, \(E = Eco\) RI, \(Ba = Bam\) HI.

![FIG 2] Autoradiograph of DNA from (A) normal subject, (B) a patient with deletion type Hb H disease, and (C) a patient with non-deletion type Hb H disease, digested with \(Bgl\) II restriction endonuclease and hybridised with the \(\zeta\) specific probe.
subjects with *Bgl* II and hybridisation with a $\alpha$-specific probe produced the 14.0 kb fragment, in addition to the normal 11.0 and 12.0 kb fragments, indicating a complement of two intact $\alpha$ globin genes ($-/-\alpha th$) (figs 1 and 2).

**CLINICAL FINDINGS**

Although consistent with the broad definition of thalassaemia intermedia, the clinical picture seen here in both groups of Hb H disease patients showed a remarkable variability. However, those patients with the [- -/(ax)th] genotype usually had the most severe clinical phenotype. Clinical presentation occurred earlier (mean age 3.2±2.0, range 0.3 to 6 years) in patients with the [- -/(ax)th] genotype than in those with the (-/- $\alpha$) genotype (mean 7.9±4, range 2.5 to 13 years). Two of the five patients with the non-deletion defect were referred to our centre because of sudden onset of a haemolytic crisis following infection or oxidative drug administration. In the remaining three with the non-deletion lesion, and in all with the deletion defect, the presentation was characterised by the slow development of a moderate anaemia (table 1).

Thalassaemia-like bone changes were seen more frequently and were more marked in patients with the [- -/(ax)th] genotype. Enlargement of the liver and spleen (lower tip more than 1 cm below the costal margin) was noted in almost all cases with the [- -/(ax)th] genotype, whereas it occurred less frequently in those with the (-/- $\alpha$) genotype (table 2). Moreover, in three out of the five patients with the non-deletion lesion the hepatosplenomegaly was quite pronounced with the lower tip 5 cm below the costal margin.

The course of the disease was more severe in those patients with the non-deletion genotype, who showed more persistent jaundice and who frequently experienced a critical episodic aggravation of the anaemia or a frank haemolytic crisis, occasionally severe enough to require blood transfusion (Hb < 5 g/dl), following infections or oxidant drug administration, but sometimes even without any specific associated event. On the other hand, patients with the deletion genotype had a milder course which was characterised by a minimal fluctuation in the intensity of the anaemia. A haemolytic crisis was observed only once in one patient in this group, who was also a carrier of the G6PD defect of the Mediterranean type.

None of the patients in either group had leg ulcers or gall stones.

**HAEMATOLOGICAL FINDINGS**

Because of fluctuating Hb levels or the development of frank haemolytic crisis, there was a marked variation in the haematological findings within the 1 to 6 years longitudinal study in both groups. Therefore, we compared the means of repeated determinations carried out at follow-up instead of the single values at presentation.

Patients with the non-deletion lesion showed more severe anaemia, significantly higher bilirubin levels, reticulocyte count, Hb H levels, and percentage of red blood cells with inclusion bodies, and significantly lower Hb A2 levels compared with those with the more usual deletion lesions.

Regardless of the genotype, both groups of patients showed $\alpha/\beta$ globin chain synthesis ratios within the range already reported in Hb H disease subjects.

There were no differences in platelet and white blood cell counts.

**Discussion**

In this study we defined by restriction endonuclease mapping the molecular defect in 14 Sardinians with

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**TABLE 1** Haematological findings in children with Hb H disease according to the molecular defect.

<table>
<thead>
<tr>
<th></th>
<th>Non-deletion type (-/-)[a]</th>
<th>Deletion type (-/-)[b]</th>
<th>$p$</th>
</tr>
</thead>
<tbody>
<tr>
<td>No of children</td>
<td>5</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>RBC (x10^12/l)</td>
<td>5.16±0.38</td>
<td>5.57±0.42</td>
<td>NS</td>
</tr>
<tr>
<td>Hb (g/dl)</td>
<td>8.4±0.9</td>
<td>9.9±0.9</td>
<td>0.02</td>
</tr>
<tr>
<td>Hct (%)</td>
<td>30.7±2.8</td>
<td>31.9±2.5</td>
<td>NS</td>
</tr>
<tr>
<td>MCV (fl)</td>
<td>61±6</td>
<td>58±3</td>
<td>NS</td>
</tr>
<tr>
<td>MCH (pg)</td>
<td>17.4±2.0</td>
<td>17.0±0.7</td>
<td>NS</td>
</tr>
<tr>
<td>MCHC (g/dl)</td>
<td>28.5±0.8</td>
<td>29.9±1.1</td>
<td>NS</td>
</tr>
<tr>
<td>Hb A2 (%)</td>
<td>0.76±0.17</td>
<td>1.31±0.30</td>
<td>0.0025</td>
</tr>
<tr>
<td>Hb H (%)</td>
<td>11.5±7.3</td>
<td>4.0±2.5</td>
<td>0.025</td>
</tr>
<tr>
<td>Hb H inclusion bodies (%)</td>
<td>573±385</td>
<td>128±114</td>
<td>0.005</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>25±16</td>
<td>28±9</td>
<td>0.0025</td>
</tr>
<tr>
<td>a/β ratio</td>
<td>0.41±0.13</td>
<td>0.37±0.11</td>
<td>NS</td>
</tr>
<tr>
<td>Transferrin saturation (%)</td>
<td>38±10</td>
<td>32±7</td>
<td>NS</td>
</tr>
<tr>
<td>Serum ferritin (ng/l)</td>
<td>94±4.5</td>
<td>53±24</td>
<td>NS</td>
</tr>
<tr>
<td>Total bilirubin (mg/dl)</td>
<td>1.38±0.61</td>
<td>0.80±0.30</td>
<td>0.05</td>
</tr>
<tr>
<td>G6PD (UI/g Hb)</td>
<td>19.2±3.4</td>
<td>12.7±6.4</td>
<td>NS</td>
</tr>
</tbody>
</table>

Statistical evaluation was carried out by Student's $t$ test.

*In two children with non-deletion type Hb H disease and in a child with the deletion defect we found 1.7%, 7.1%, and 1.8% Hb Bart's, respectively.

*One child with deletion type Hb H disease had a G6PD defect of the Mediterranean type.

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**TABLE 2** Clinical findings in children with Hb H disease according to the molecular defect.

<table>
<thead>
<tr>
<th></th>
<th>Non-deletion type (-/-)[a]</th>
<th>Deletion type (-/-)[b]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presentation with a haemolytic crisis</td>
<td>2/5</td>
<td>0/9</td>
</tr>
<tr>
<td>Thalassaemia-like bone changes</td>
<td>2/5</td>
<td>2/9</td>
</tr>
<tr>
<td>Persistent slight jaundice</td>
<td>4/5</td>
<td>3/9</td>
</tr>
<tr>
<td>Spleen enlargement</td>
<td>4/5</td>
<td>2/9</td>
</tr>
<tr>
<td>Liver enlargement</td>
<td>4/5</td>
<td>3/9</td>
</tr>
</tbody>
</table>

[Discussion]
the clinical and haematological phenotype of Hb H disease. In nine of the 14 patients, the underlying molecular defect was found to be the deletion of three of the four α globin structural genes (-/-/α). In the remaining five we found a combination of the deletion of two α globin genes in one chromosome (-/-) with a non-deletion lesion [(αα)hb], as yet incompletely characterised, in the other. We then compared the clinical and haematological phenotypes of these two groups of patients with Hb H disease resulting from these different molecular mechanisms. Our results clearly show that the Hb H disease phenotype produced by the combination between the deletion (-/-) and non-deletion [(αα)hb] determinants is more severe than that resulting from the more common deletion genotype (-/-/α). Those patients with the non-deletion lesion, in fact, presented earlier with a moderate anaemia or with a frank haemolytic crisis. At follow-up, in those patients with the non-deletion lesion, thalassaemia-like bone changes and enlargement of the liver and spleen were observed more frequently and were more marked. Furthermore, the course of the disease was more severe in patients with the non-deletion lesion because of episodic worsening of the anaemia or development of frank haemolytic crises. Accordingly, mean Hb levels from repeated determinations carried out over the period of follow-up were lower and bilirubin levels higher in those patients with the non-deletion defect.

Such clinical effects could well result from an increased red blood cell destruction rate in the spleen, or more intense ineffective erythropoiesis in the bone marrow of patients with the non-deletion as compared to those with the deletion lesion, or both. Although the large gap in our knowledge about the pathophysiology of Hb H disease precludes giving a precise outline of the mechanisms underlying these clinical differences, from our results it seems that patients with the non-deletion defect have a more severe shortage of α globin chains resulting, in turn, in a raised mean level of Hb H in peripheral blood and in a higher percentage of cells with inclusion bodies, which may be the cause of the reduced life span of the red blood cells.

One puzzling feature, however, of the effects of the non-deletion defect is the consistently higher Hb H levels in spite of a similar imbalance of globin chain synthesis as compared to the deletion lesion. However, as clearly stated by Weatherall and Clegg,3 there appears to be as yet undefined factors, other than the degree of chain imbalance, which determine the final level of Hb H in peripheral blood. Despite a similar degree of globin chain imbalance, in fact, in a large series of patients, the level of Hb H showed a remarkable variation, ranging from 2 to 40%.5

Our findings indicate that the Sardinian non-deletion determinant described here results in a more severe phenotype than the α+-thalassaemia deletion defect, but it is obviously milder than the α-thalassaemia lesion, as the non-deletion determinant does not result in a complete suppression of α globin chain production. Accordingly, in relatives of these patients, the presence of the [(αα)hb] determinant was found to lead to a significant reduction in MCH and MCV values as compared with those of persons carrying the (-α) determinant (unpublished results). Therefore, although both genes are intact on the chromosome, this lesion appears to result in a more pronounced reduction of α globin chains than that produced by the single α gene deletion defect.

A non-deletion determinant with a mRNA output equivalent to about half that of a single gene, producing Hb H disease in the homozygous state, has recently been described by Pressley et al10 in Saudi Arabs. This observation suggests that the Saudi Arabian determinant has a more severe phenotypic effect than the Sardinian non-deletion one. As has already been seen in other types of thalassaemia, the non-deletion α-thalassaemia lesion is probably highly heterogeneous even in the same population.8 Characterisation of the molecular defects in other Hb H disease patients should allow for more accurate phenotype-genotype correlations and thereby elucidate the molecular basis of the extraordinary clinical variability of this type of thalassaemia intermedia.

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