Haptoglobin Polymorphism and Iron Homeostasis
Ernest Beutler,* Terri Gelbart, and Pauline Lee

Background: There is a marked difference in the degree of expression of the homozygous C282Y HFE genotype that is associated with hereditary hemochromatosis. It has been reported that individuals with the haptoglobin 2-2 type manifest increased iron concentrations, including serum iron, transferrin saturation, and ferritin.

Methods: We studied 232 patients, 115 homozygous for the c.845G→A (C282Y) mutation and 117 matched controls with the wild-type HFE genotype, for haptoglobin phenotypes. Haptoglobin types were determined by electrophoresis of the denatured protein. The HFE genotype was determined by allele-specific oligonucleotide hybridization. Ferritin and transferrin saturation were measured by standard methods.

Results: There was no relationship between haptoglobin type and ferritin concentration or transferrin saturation.

Conclusions: The effect of haptoglobin type on iron homeostasis cannot account for the marked phenotypic variation that is seen in patients homozygous for the HFE C282Y mutation.

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The c.845G→A (C282Y) mutation of the HFE gene is common, but of the many homozygotes in the Northern European population, ~5 per 1000, only very few develop clinical hemochromatosis. Indeed, only approximately one-half manifest increased ferritin concentrations, even when past middle age (1, 2). Clearly, other factor(s) determine whether clinical hemochromatosis will develop in homozygotes.

Recently, groups in France and Belgium have reported that a common haptoglobin polymorphism is a risk factor in men homozygous for the C282Y HFE mutation and in men from the general population (3–5). It was suggested that serum iron, transferrin saturation, and serum and monocyte ferritin concentrations were increased in men with the 2-2 haptoglobin phenotype. We have typed 232 patients, 115 homozygous for the c.845G→A (C282Y) mutation and 117 matched controls who have the wild-type/wild-type (wt/wt) genotype. We have been unable to find any relationship between the haptoglobin type and iron concentrations.

Materials and Methods

Patients
The patient population and methods for the determination of serum ferritin and transferrin saturation have been described previously (1, 2, 6). Briefly, these patients attended the Health Appraisal Clinic at Kaiser-Permanente in Southern California. HFE genotyping was performed on >41 000 individuals who volunteered to participate in a study of hemochromatosis. Only those homozygous for the C282Y mutation for whom serum ferritin and transferrin saturation values were available before having undergone phlebotomy were included. Table 1 summarizes the demographic characteristics of the patient sample. The controls were homozygous wild-type individuals, matched to the C282Y/C282Y homozygotes by sex, age, and ethnic origin for the determination of serum collagen IV in a previous study (2). For a few participants, plasma was not available for genotyping.

Haptoglobin Typing
Type 2 haptoglobin is the product of a gene with a major internal duplication; the haptoglobin type can therefore be determined on the basis of the molecular mass by electrophoresis of the α-chain of the denatured protein. A modification of the method of Craig et al. (7) was used. We diluted 1 µL of human EDTA plasma that had been stored for up to 2 years at −70 °C in 20 µL of sample loading buffer [10 g/L sodium dodecyl sulfate, 100 mL/L glycerol, 25 mmol/L Tris (pH 6.8), 0.05 g/L bromphenol blue, and 50 mL/L β-mercaptoethanol] and boiled the mixture for 5 min. We then loaded 5 µL of the boiled sample on a precast 15% polyacrylamide gel (9 × 10 cm) from BioWhittaker. Standard haptoglobin samples of types 1-1 and 2-2 (Sigma Chemical Co.) were diluted to 1 g/L and treated in the same fashion. Samples were electrophoresed in 25 mmol/L Tris base–192 mmol/L glycine–¡ g/L sodium dodecyl sulfate for 45 min at 150 V and then transferred to Protran® (Schleicher and Schuell) nitrocellulose membranes. The membranes were blocked...
in 20 g/L bovine serum albumin in Tris-buffered Tween [TBST; 10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 0.5 mL/L Tween 20] for 1 h and then incubated at 4°C overnight with a 1:1000 dilution of polyclonal rabbit anti-human haptoglobin (Sigma) as a primary antibody. After washing the membrane three times in TBST, we added a second antibody, anti-rabbit IgG Fc alkaline phosphate conjugate (Promega) diluted 1:7500 in TBST; the membrane was then incubated at room temperature for 1 h. The membrane was washed three times in TBST and finally developed with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate color development substrate (Promega).

**Results**

As shown in Fig. 1, α- and β-haptoglobin bands were visible after incubation with polyclonal rabbit anti-human haptoglobin. The α bands determine the 1-1, 1-2, and 2-2 haptoglobin types.

The validity of the immunoblotting method for determining haptoglobin genotypes was confirmed by examining genomic DNA from the same individual by multiplex PCR. Two different sense primers, one common to haptoglobin types 1 and 2, the other specific only to haptoglobin type 2, were used with a common antisense primer:

- Sense primers: type 1, 5′-CTGCCAGAAATGAGGG-GAGCT-3′; type 2, 5′-GAGCTCCAGGCCAGTGTCT-3′

Antisense primer: 5′-TTCTTACACTGGTAGCGAACC-G-3′

The DNA from those individuals who were haptoglobin type 1 yielded only a 225-bp PCR fragment. The DNA from individuals who were haptoglobin type 2 yielded two bands, 225 and 332 bp in length, that were of equal intensity. The DNA from individuals heterozygous for type 1 and 2 also yielded two bands of 225 and 332 bp, but the intensity of the 225-bp band was greater because it is represented in both haptoglobin type 1 and type 2, whereas the 332-bp band is amplified only from haptoglobin type 2. The Western blot-based method was chosen as the least error-prone because evaluation of intensity of bands was not required for interpretation. However, in every case in which both methods were used, the same result was obtained.

The distribution of haptoglobin types in the population studied is shown in Table 2. There was no significant difference in the gene frequencies in the various subgroups studied, nor was there significant deviation from the expected Hardy–Weinberg distribution for the genotypes.

The relationship between serum ferritin and transferrin saturation in the different haptoglobin types among the men is shown in Fig. 2. The values in women were somewhat lower, but there was again no significant difference between different haptoglobin types (data not shown).

**Discussion**

It is not known whether the factors that determine which homozygotes for the C282Y mutation develop clinical disease are primarily genetic or environmental. On the basis of the presumption that genetic polymorphisms may be important modifiers of the expression of HFE mutations, we have examined polymorphisms encoding 15 genes involved in iron homeostasis, utilizing a large database obtained in Southern California. Although we found that one of the polymorphisms of the transferrin gene is a risk factor for iron deficiency anemia in women (10), no other polymorphism was found that significantly increased the transferrin saturation or serum ferritin concentrations of either homozygotes for the C282Y mutation or patients with a wt/wt HFE genotype.

Haptoglobin serves to scavenge hemoglobin that escapes into the plasma and carries it to the liver parenchyma (11) and to macrophages (12). There is no a priori

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**Table 1.** Demographic characteristics of the population studied.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sex</th>
<th>Age, <em>years</em></th>
<th>White</th>
<th>Hispanic</th>
<th>Mixed/Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>C282Y/C282Y</td>
<td>M</td>
<td>56.88 ± 13.26</td>
<td>53</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>57.28 ± 14.02</td>
<td>49</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>wt/wt</td>
<td>M</td>
<td>57.15 ± 11.95</td>
<td>56</td>
<td>4</td>
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</tr>
<tr>
<td></td>
<td>F</td>
<td>57.09 ± 14.33</td>
<td>50</td>
<td>5</td>
<td>2</td>
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</table>

* Mean ± SD.
reason to believe that the haptoglobin pathway is an important limiting factor in iron homeostasis. One reason is that the vast majority of hemoglobin iron does not pass through this pathway; it has been estimated that the flow that can be attributed to free hemoglobin represents only 10% of the total hemoglobin normally catabolized (13), and this may well be an overestimate because it depends on measurement of plasma hemoglobin concentrations, which usually contain a substantial artifactual component. Another reason is that major disturbances of iron metabolism have not been reported in patients with anhaptoglobinemia attributable to a deletion of a portion of the haptoglobin cluster on chromosome 16q22 (14, 15). Moreover, the haptoglobin knockout mouse (16) has not been reported to have any abnormality of iron homeostasis, and healthy infants generally have no detectable haptoglobin (17). However, there is a recent body of evidence suggesting that there is a relationship between the haptoglobin type and serum ferritin concentrations (3–5).

It has been proposed that in patients homozygous for the C282Y mutation, transferrin saturation and ferritin concentrations are higher in those with the haptoglobin 2-2 type, although there seems to be no greater prevalence of liver fibrosis. In the present investigation we were unable to confirm these results in our population of patients. Indeed, the ferritin concentrations were actually lower, albeit not significantly so, in 2-2 homozygotes than in patients with other haptoglobin types. This was true even when the analysis was limited to the white patients in the sample (data not shown). The population of patients we studied was based on patients who had been diagnosed on the basis of their genotype, not because of biochemical changes or clinical stigmata of the disease. In contrast, the studies performed by others were based on patients who had been diagnosed as having hemochromatosis, presumably largely because of increased serum transferrin saturation and ferritin concentrations. Because our population was more diverse, it permitted a more incisive examination of the question of whether there is a true relationship between iron concentrations and the haptoglobin phenotype, the patients with low iron within the homozygous group serving as controls for those with high iron. However, we detected no difference.

There is no ready explanation for the discrepancy between our results and those reported by other authors, although it is possible that the putative effects of haptoglobin type are actually attributable to linkage disequilibrium with other genes or to population stratification with respect to ethnic origin. It is of interest, in this regard, that no relationship between iron concentrations and haptoglobin phenotype could be found in an African population (18). In any case, the effect must be a minor

<table>
<thead>
<tr>
<th>Sex</th>
<th>HFE genotype</th>
<th>Haptoglobin</th>
<th>1-1</th>
<th>1-2</th>
<th>2-2</th>
<th>Total</th>
<th>1</th>
<th>2</th>
</tr>
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<tr>
<td>M</td>
<td>C282Y/C282Y</td>
<td>10</td>
<td>27</td>
<td>20</td>
<td>57</td>
<td></td>
<td>0.412</td>
<td>0.588</td>
</tr>
<tr>
<td>M</td>
<td>wt/wt</td>
<td>9</td>
<td>35</td>
<td>16</td>
<td>60</td>
<td></td>
<td>0.442</td>
<td>0.558</td>
</tr>
<tr>
<td>F</td>
<td>C282Y/C282Y</td>
<td>8</td>
<td>31</td>
<td>19</td>
<td>58</td>
<td></td>
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<td>57</td>
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<tr>
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<td>36</td>
<td>120</td>
<td>76</td>
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<td></td>
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*gf* is the gene frequency.

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![Fig. 2. Relationship between haptoglobin type and serum ferritin (top) and transferrin saturation (bottom) in males.](image)
one and cannot explain the large difference in the homozygous C282Y phenotype.

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References