The Hematologic Characteristics of Sickle Cell Anemia Bearing the Bantu Haplotype: The Relationship Between $\gamma^\alpha$ and HbF Level

By Ronald L. Nagel, Srinivas K. Rao, Olga Dunda-Belkhodja, Mary M. Connolly, Mary E. Fabry, Alan Georges, Rajagopal Krishnamoorthy, and Dominique Labie

Present evidence suggests that the HbS gene appeared in Africa at least three times and presumably expanded in frequency through the selective pressure of *Plasmodium falciparum* malaria. The best evidence for this hypothesis comes from the geographically segregated linkage disequilibrium of the HbS gene with three different $\beta$ gene cluster haplotypes (the Senegal, the Benin, and the Bantu haplotypes). These are indeed the major haplotypes associated with the HbF gene and can be confirmed by the study of Jamaican and American sickle cell anemia (SS) patients. This finding suggested the possibility that the HbS gene could be accompanied by a different set of linked epistatic genes in each of these three haplotypes. Indeed, we have reported previously that SS patients bearing the Senegalese haplotype have more HbF, less dense cells, and higher $\gamma^\alpha$ expression than patients in which the HbS gene is linked to the Benin haplotype. We report here on the hematologic and genetic characteristics of the third haplotype, the Bantu, and a comparison with the other two reveals that it is associated with yet another unique set of features.

**METHODS**

Samples were obtained, after informed consent, from all the unrelated attendants of the outpatient clinic who came without regard to present symptomatology after a public announcement. Patients with a history of painful crisis less than a week old were eliminated from the sample. The hematologic parameters were determined as previously described. $\beta$ Gene cluster and $\alpha$ gene haplotypes were determined by Southern blotting in the manner reported elsewhere by using the HindIII detectable polymorphic site in the IVS1 region of the $\gamma$ genes, the HindII sites in the $\beta$ gene region, and HpaII site $\beta$ to the $\beta$ gene. In a subset of samples in addition, the HindII site $\beta$ to $\beta$, XmaI $\beta$ to $\gamma$, the PvuII site $\beta$ to $\delta$, the Avall in IVSII of the $\beta$ chain and the BamHI $\gamma$ to the $\delta$ site were determined. The $\alpha$ haplotype was determined by BamHI digestion. Percoll-Stratracian isopycnic gradients were made as previously described, and the four classes of cells were defined as fraction 1 (F1, mean corpuscular hemoglobin concentration [MCHC] less than 33 g/dL, densities less than 1.083 g/mL), fraction 2 (F2, MCHC between 33 and 37 g/dL, densities between 1.083 and 1.093 g/mL), fraction 3 (F3, MCHC between 37 and 42 g/dL, densities between 1.093 and 1.105 g/mL), and fraction 4 (F4, MCHC greater than 42 g/dL, densities greater than 1.105 g/mL). F1 contains reticulocytes and young cells, F2 reversible discocytes, F3 the unsickled SS discocytes, and F4 the densest irreversible discocytes and the irreversibly sickled cells (ISC).

Percent HbF present as $\gamma^\alpha$ was determined by a high-performance liquid chromatography method using a Vydac large-pore (300 Å) C4 column, 4.6 x 250 mm (Separations Group, Hesperia, CA) and an acetonitrile/trifluoroacetic gradient. The HbF was purified by BioRex-70 (Bio-Rad, Richmond, CA) column chromatography. Statistics were done with the help of an IBM-XT computer and the Statgraphics program (STSC-Plus-Ware, Rockville, MD) for plotting, frequency histogram, regression analysis, the two-sample $t$ test, and cluster analysis.

**RESULTS**

We have studied 64 unrelated SS patients from the Central African Republic. Of these 128 chromosomes, 116 $\beta$ genes were linked to the Bantu haplotype (90.6%), whereas 12 chromosomes were atypical and their characteristics will be reported elsewhere.

Hematologic and genetic characteristics of SS-bearing Bantu haplotype. We report here on the hematologic and genetic characteristics of 54 SS patients (mean age, 14 ± 6.3 SD) in whom the Bantu $\beta$ gene cluster haplotype has been demonstrated by defining five polymorphic sites. Of these 54 patients, 36 were females and 18 males, 26 were $\alpha\alpha/\alpha\alpha$...
(48.4%), 22 were $-\alpha/\alpha\alpha$ (40.6%), and 6 were $-\alpha/-\alpha$ (11.1%). The gene frequency of the $-\alpha$ haplotype is then 0.315 among these patients, higher than that found among AA individuals in the Central African Republic, which predicts that about 62% of patients with SS have concomitant $\alpha$-thalassemia. This result is in agreement with earlier data that found the frequency of $-\alpha$ increased among sickle cell anemia patients as compared with the normal population, which suggests a protective effect of $\alpha$-thalassemia in terms of survival. This suggestion has been confirmed in several populations by the age dependence of the $-\alpha$ gene frequency, although the data of others have only partially confirmed this finding by observing four homozygotes among the patients over 50 years old.

In Table 1 the hematologic and genetic characteristics of the total sample and of the sample separated according to $\alpha$ gene status are depicted. Prominent among the findings are the high percentage of HbF (10.4%) and the corresponding low percentage of F4 (8.9%) and ISC (8.7%). The HbF levels are significantly correlated with the level of F4 ($r = .320, P < .018, n = 54$) as previously demonstrated for SS patients with the Senegalese haplotype.$^4$ HbF also correlated with ISC ($r = .299, P < .027$), not surprisingly since the percentage of ISC in the total population correlates with the percentage of F4 very strongly ($r = .863, P < .4 \times 10^{-13}$). It is interesting to note that the correlation of the percentage of ISC $v$ F4 had an $r^2$ of .86 for $\alpha\alpha/\alpha\alpha$ individuals but that the $r^2$ for $-\alpha/\alpha\alpha$ and $-\alpha/-\alpha$ patients was much lower (.32), which suggests that in concomitantly $\alpha$-thalassemic individuals the percentage of ISC is a less accurate reflection of the percentage of dense cells.

The reticulocyte count was correlated with F4 in the total sample ($r = .304, P < .025$). The hematocrit (Hct) value was marginally correlated with the percentage of F4 ($r = .26, P < .05$) and the reticulocyte count ($r = .26, P < .05$).

The $G_\gamma$ ratio was 40.4% (±6.2 SD) in 54 patients studied, and no effect of $\alpha$-thalassemia on this parameter is observed.

Table 2 compares the hematologic properties of the Bantu haplotype SS with the Senegalese and Beninians. The Bantu and the Senegalese share a high percentage of HbF and a low percentage of F4, even when the elevated frequency of $\alpha$-thalassemia in the Bantu population is taken into account by comparing the non-$\alpha$-thalassemic Bantu to the Senegalese in whom $\alpha$-thalassemia is less prevalent. Nevertheless, they are different in that the Senegalese haplotype is linked to high $G_\gamma$ expression and the Bantu is linked to low $G_\gamma$ expression in the adult. Both haplotypes are in turn very different from the Beninian haplotype, which is characterized by low HbF and high F4 percentages.

The percentage of HbF among the Bantu is significantly different from the Beninian ($P < .009$), as are the percentage of F4 ($P < .00002$) and the percentage of ISC's ($P < .005$). The Bantu percentage of F4 was also significantly different from the Senegalese frequency of F4 ($P < .05$).

The distribution of the percent HbF among patients with sickle cell anemia is also different for the three haplotypes (Fig 1). The mode for the percentage of HbF among patients with the Benin SS haplotype is in the class between 2.6% and 5%, with a nongaussian distribution trailing off to higher percentages. In contrast, the mode for Senegal and Bantu haplotypes is 10.1% to 12.5%, although a higher proportion (33% v 25%) of Senegalese are found with a percentage of HbF greater than 10. The difference between these two groups is the lack of Senegalese SS with a HbF below 5%, a class that is populated by over 11% of the Bantu SS patients.

The relationship between percent HbF and percent $G_\gamma$. Figure 2 illustrates the relation between the percentage of $G_\gamma$ and the percentage of HbF for the patients bearing the Bantu haplotype and a comparison with the Senegalese and Beninian sickle cell anemia patients. Not all Senegalese SS are known to carry the Senegalese haplotype. For the Bantu SS restricted to individuals 5 years of age or older ($n = 52$), the

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All SS</th>
<th>SS + $-\alpha/\alpha\alpha$</th>
<th>SS + $-\alpha/-\alpha$</th>
<th>SS + $-\alpha/-\alpha$</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>54</td>
<td>26</td>
<td>28</td>
<td>6</td>
</tr>
<tr>
<td>Sex, percent female</td>
<td>66.6</td>
<td>66.6</td>
<td>75.0</td>
<td>66.6</td>
</tr>
<tr>
<td>Age</td>
<td>14.0 ± 6.3</td>
<td>12.6 ± 6.8</td>
<td>15.4 ± 5.7</td>
<td>14.7 ± 8.4</td>
</tr>
<tr>
<td>Hct</td>
<td>23.8 ± 3.7</td>
<td>23.1 ± 3.3</td>
<td>24.5 ± 3.9</td>
<td>24.8 ± 4.0</td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>25.0 ± 13.1</td>
<td>25.5 ± 13.7</td>
<td>24.8 ± 12.8</td>
<td>19.6 ± 9.9</td>
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<td>HbF (%)</td>
<td>10.4 ± 4.8</td>
<td>10.35 ± 5.1</td>
<td>10.4 ± 4.7</td>
<td>10.8 ± 0.7</td>
</tr>
<tr>
<td>$G_\gamma$ (%)</td>
<td>40.4 ± 6.2</td>
<td>42.6 ± 5.0</td>
<td>39.2 ± 7.4</td>
<td>37.5 ± 1.4</td>
</tr>
<tr>
<td>ISC</td>
<td>8.7 ± 9.4</td>
<td>8.8 ± 12.0</td>
<td>8.5 ± 6.1</td>
<td>7.5 ± 4.1</td>
</tr>
<tr>
<td>F1*</td>
<td>46.9 ± 22.5</td>
<td>50.5 ± 26.3</td>
<td>43.5 ± 18.2</td>
<td>43.7 ± 24.9</td>
</tr>
<tr>
<td>F2</td>
<td>39.7 ± 19.2</td>
<td>34.9 ± 21.2</td>
<td>44.1 ± 16.2</td>
<td>45.8 ± 22.3</td>
</tr>
<tr>
<td>F3</td>
<td>4.5 ± 3.3</td>
<td>4.7 ± 3.7</td>
<td>4.4 ± 2.9</td>
<td>4.4 ± 3.1</td>
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<tr>
<td>F4</td>
<td>8.9 ± 8.0</td>
<td>9.9 ± 10.5</td>
<td>7.9 ± 4.7</td>
<td>6.0 ± 3.0</td>
</tr>
</tbody>
</table>

Numbers (except for n = number of patients and sex) are mean ± SD.

*F1, F2, F3 and F4 are density-defined red cell SS fractions obtained from Percoll-Stractan isopycnic gradients.
The presence or absence of α-thalassemia does not affect the results. Statistical cluster analysis of the data in Fig 2A reveals that there is a cluster of patients with less than 38% Gγ, scarcely represented among Beninian patients (2B). When the percent HbF in the group of Bantu SS patients with less than 38% Gγ is compared with the percent HbF in those with more than 38.1%, the two-sample t test is highly significant, with a mean of 6.34 ± 2.46, and 12.46 ± 4.0, respectively, (t = 5.909, DF = 50, P < 3.0 x 10^-7 (Table 3). In addition, the variance in the percent HbF among those above 38.1% Gγ is much higher than that in those below 38% Gγ. The presence or absence of α-thalassemia, again, does not affect these findings. No differences were observed, and all subjects were Xmn I-negative.

Most interesting are the calculations of regression curves below and above 38% Gγ. The r value for a correlation between HbF v ISC or F4 is (not significant) in the subset equal or less than 38% Gγ, whereas it is highly significant (for ISC = r = -.53, DF = 29, t = -3.32, P < .005; and for F4 = r = -.45, DF = 29, t = -2.79, P < .01) among those equal or higher than 38.1% Gγ.

DISCUSSION

One of the most perplexing features of sickle cell anemia is the extreme variability of the hematologic characteristics and severity of its symptomatical components when a large number of patients are analyzed. It has recently become clear that one of the sources of this heterogeneity is the presence of the Hbs gene on chromosomes bearing at least three different arrays of linked genes and sequences, which arose and expanded in geographically segregated regions of Africa and generated three different haplotypes linked to these Hbs genes. The Senegalese haplotype is found in Senegal and surrounding areas of Atlantic West Africa; the Benin haplotype is found in Benin and surrounding areas of Central West Africa and in North Africa and the Mediterranean where its presence has been extended through gene flow; and finally, the Bantu haplotype is found in the Central African Republic and presumably (on the basis of HpaI polymorphism data) in the whole of the Bantu-speaking equatorial and Southern Africa.

To what extent do these different haplotypes linked to Hbs generate different hematologic, genetic, and clinical features? Are there three types of sickle cell anemia?
SICKLE CELL–BANTU HAPLOTYPE: HbF AND αγ

Table 3. Hematologic Parameters in SS Patients with Bantu Haplotype (≥5 Years Old) Divided into Two Subsets by 0γ Level

<table>
<thead>
<tr>
<th>Parameter</th>
<th>&lt;38% 0γ*</th>
<th>Mean ± SD</th>
<th>Variance</th>
<th>&gt;38.1% 0γ†</th>
<th>Mean ± SD</th>
<th>Variance</th>
</tr>
</thead>
<tbody>
<tr>
<td>HbF (%)</td>
<td>6.3 ± 2.5</td>
<td>6.1</td>
<td>12.5 ± 4.0</td>
<td>16.0‡</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hct (%)</td>
<td>25.2 ± 3.6</td>
<td>13.2</td>
<td>23.0 ± 3.7</td>
<td>13.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reticulocytes (%)</td>
<td>26.1 ± 13.9</td>
<td>192.7</td>
<td>25.3 ± 13.3</td>
<td>177.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>F4 (%)</td>
<td>8.1 ± 5.5</td>
<td>30.6</td>
<td>9.6 ± 9.3</td>
<td>86.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>ISC (%)</td>
<td>7.8 ± 6.0</td>
<td>36.1</td>
<td>9.7 ± 11.2</td>
<td>125.9</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*7(α/αα), 9(−α/αα), 2(−α/−α); n = 18.
†20(αα/αα), 14(−α/αα); n = 34.
‡P < .0000003.

We have partially answered this question, previously, by studying haplotypically homozygous SS patients bearing the Senegalese and the Beninian haplotypes. That study demonstrated that, indeed, the Senegalese haplotype linked to the HbS was characterized by higher HbF levels, a lower percentage of dense cells, and a high 0γ expression in the adult. That their features are genetic in origin is given considerable support by the finding that the same haplotype induces a similar HbF response in thalassemia even though this disease is under different pathophysiological control. In contrast, the Beninian haplotype was characterized by SS patients exhibiting lesser amounts of HbF, a larger proportion of dense cells, and low 0γ gene expression. A model that describes the combined effect of α-thalassemia and percent HbF on percent F4 (dense cells) was developed for our New York black population and for the Senegalese and Beninian populations. The relative contribution of α-thalassemia and percent HbF was different for each population as would be anticipated if there were a third factor (haplotype) that also affects dense cell generation.

The characteristics of SS patients bearing the Bantu haplotype have features that distinguish them from the other two haplotypes. First, the mean and mode (Table 2 and Fig 1) of the percentage of HbF is high and similar to the SS patients with Senegalese haplotypes. Nevertheless, the distribution of the values is different: no SS patient with Senegalese haplotype had an HbF level less than 5%, whereas 11% of Bantus had an HbF level between 0% and 5%, although mostly between 2.6% and 5% (Fig 1). The situation is considerably different among the patients bearing the Benin haplotype who exhibit a HbF distribution with a mode between 2.6% and 5% and a very nongaussian distribution with a long tail toward the higher values. Another important feature of the Bantu sickle cell anemia patient is their low percentage of dense cells, again similar to the Senegalese and not exclusively due to higher frequency of α-thalassemia among the Bantu. Although there is a significant correlation between percent F4 or percent ISC and percent HbF in both populations, a lesser proportion of the variability can be explained by the level in the percentage of HbF among the Bantu than in the Senegalese (r² = .53 ± .10). However, if only the non−α-thalassemic Bantu patients are considered, r² rises from .10 to .22 with a P > .02.

A further characteristic that differentiates the Senegalese from the Bantu is the 0γ expression. The Senegalese exhibit a high 0γ expression among adults, an infrequent finding among normal individuals who switch from the newborn 70% 0γ/30% 4γ ratio to the adult 40% 0γ/60% 4γ ratio during the first 4 months of life. The Bantu SS have the low 0γ expression (Table 1) characteristic of adults.

Although the genetic linkage between HbS and haplotypes and between haplotypes and 0γ expression is well established, no formal linkage analysis of the percentage of HbF and haplotype has yet been possible. Although the statistically significant association between the mean and frequency distribution of the percentage of HbF and haplotype suggests genetic linkage, it is nevertheless already clear that modulating epistatic effects from other genes or factors will result in a not very tight linkage. Family studies, hampered by the absence of the anemia-induced HbF response in parents, will be needed to quantify the linkage.

The next question is whether there is a correlation between percent 0γ and percent HbF. Previous studies of American SS patients over the age of 5 have found no significant correlation between these two parameters (although a weak correlation was found in SS patients below this age), and the same is true of the previously studied Beninian SS patients (Fig 2). On the other hand, among SS patients bearing the Bantu haplotype there is a significant correlation (P < .0001) between 0γ and HbF that, on further analysis, seems to arise from a cluster of individuals with a low expression of 0γ (<38%). This group is little represented among the Senegalese, the Beninians, and the patients analyzed by Huisman et al. The low 0γ subset is associated with a low percentage of HbF, whereas the subset with higher 0γ expression has a significantly higher percentage of HbF (Table 3). Interestingly, the variance is low in the former and large in the latter. These findings explain why no significant correlation has been found in populations of patients in which the group with low 0γ expression is not well represented. In addition, if the data of the Huisman group is divided into less than and more than 38% 0γ, a significantly lower percentage of HbF is observed for the <38% 0γ subset: for patients who are 5 years old or older, the mean is 3.87 ± 3.3 compared with 12.2 ± 5.5 for the >38.1% 0γ (P < .01).

The low 0γ subset exhibits poor correlation between HbF and F4 or ISC, whereas the higher than 38.1% subset exhibits a strong negative correlation (r = .53, P < .005). This dimorphic population explains the lower correlation of HbF and dense cells in the overall sample.

The heterogeneity in 0γ expression among the Bantu and its relation to HbF expression is a significant finding that...
opens new avenues of investigation. Our working hypothesis is that the subset with lower than 38% 6γ corresponds to a distinct chromosome bearing different HbF determinants than the rest of Bantu haplotypes (although not distinguishable by the ten endonuclease-definable polymorphic sites including the XmnI site). Efforts to define these determinants by sequencing are underway in our laboratory.

A complementary explanation is that the enrichment factor (the ratio between F cells and F reticulocytes14) might modulate the HbF level, particularly among individuals with a percentage of 6γ between 38 and 50. This last effect might be the consequence of the dependence of the enrichment factor on the constitutive HbF expression (percentage of HbF reticulocytes), a possibility supported by the data of Dover et al.14 These variations also might affect the resulting percentage of HbF more significantly in higher HbF-producing individuals. In addition, the variability of dense cells with respect to HbF level might depend on the distribution function of HbF among HB5-containing cells, a factor that may be genetically determined.

We conclude that the Bantu haplotype results in yet another set of hematologic and genetic characteristics that exhibit a high mean HbF percentage (although with a different distribution than the Senegalese), a low percentage of dense cells (although less determined by the HbF level than among Senegalese), and a low 6γ expression (unlike the high 6γ expression characteristic of the Senegalese) but with a wide range of the 6γ percentage (unlike the distribution among Beninians).

Because patients in the Americas originated from the three geographically segregated but haplotypically homogeneous areas of Africa,15 they can be expected to belong to the three types of sickle cells anemia, but it is further complicated by both non-African admixture and by the presence of a high number of haplotypically heterozygous individuals whose hematologic characteristics have not been defined.

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