The Heterogeneity of the γ-Chain of Fetal Hemoglobin in HbS Heterozygotes

By M. B. Gardiner, A. L. Reese, M. E. Headlee, and T. H. J. Huisman

The relative quantities of the three types of γ chain (γ,γ',γ'') were determined in 18 AS types of selected SS patients, in 15 additional HbS heterozygotes, as well as in additional SS patients, and in 35 SS and 24 AS newborn babies. The low amount of HbF in all AS adults (≤ 1%) made it necessary to further improve the isolation procedure of HbF, which was accomplished by introducing an HPL chromatographic method. The additional data for older SS patients confirmed the existence of two groups characterized by either low γ (40%) or high γ (60%) values in their HbF. A distinction into "high γ" and "low γ" producers could also be made for HbS heterozygotes. Family studies, however, make it unlikely that the "high γ" condition is inherited in a simple mendelian fashion assuming a change in a regulatory mechanism. The presence of the γ mutation, occurring either in cis or in trans to the β mutation, has been used to evaluate the possible contribution by specific γ genes to the γ-chain composition of the HbF of adult AS persons. No clear pattern became evident, suggesting that most of the γ-chain of this small amount of HbF could originate from γ-chain genes in cis or in trans to the β gene or from both sets of genes. It is speculated that heterogeneity among "F-cells" may be a primary cause of the observed differences in γ-chain composition of HbF in HbS heterozygotes.

The three types of γ-chain of human fetal hemoglobin (HbF), i.e., 5γ (136 Gly, 75 Ile), 5γ' (136 Ala, 75 Ile), and 5γ'' (136 Ala, 75 Thr), have been observed in several racial and ethnic groups. The 5γ-γ' chain heterogeneity is universal, while the 5γ'' chain, which is a variant of the 5γ' chain, occurs in varying frequencies in different populations. Recently developed micromethods and high-pressure liquid chromatography (HPLC), in particular, have greatly simplified the quantitation of the three types of γ-chain. As a result, new data describing the γ-chain heterogeneity in normal adults, thalassemic patients, subjects with various types of the hereditary persistence of fetal Hb (HPFH), sickle cell anemia (SS) patients, and normal newborns and babies with various hemoglobin abnormalities have appeared.

The HbF of SS patients, 6 yr old, contains γ and γ' chains in a constant ratio of either 4:6 or of 6:4, with that the switch from the newborn ratio of 7:3 to these adult ratios appears to be delayed. There is considerable evidence that the "high γ" and "low γ" values in these patients are genetically determined, since they occur in families. This aspect was further investigated through analyses of the HbF from HbS heterozygous relatives, and the results of these studies are reported in this article. The selection of these persons was in part determined by the possible presence or absence of the γ'' chain because such an additional genetic marker might facilitate the interpretation of the quantitative data.

MATERIALS AND METHODS

Blood Samples

Samples were obtained from numerous adult and pediatric SS patients attending the two outpatient clinics of the Comprehensive Sickle Cell Center in Augusta. Several patients were routinely followed in these clinics, but in about one-third of them the diagnosis was established for the first time with procedures in use in our laboratories. In addition, cord blood samples from 35 SS and 24 AS newborn babies were studied. The diagnosis of sickle cell anemia and HbS trait in these babies was based on data obtained by cellulose acetate electrophoresis at pH 8.6 and citrate agar electrophoresis at pH 6.1, and by a newly developed HPLC chromatographic method that allows a complete separation of the hemoglobins A, S, and F present in cord blood red cell lysates. Thirty-three adult HbS heterozygotes participated in this study. Eighteen were parents of selected SS patients, while 15 were relatives of patients with other hematologic abnormalities who were also heterozygous for the γ'' chain. Only a small volume (1-3 ml) of cord blood was available, while 2-10 ml of blood was collected from each SS patient. The HbS heterozygotes donated 20 ml of blood. All blood samples were collected in vacutainers with EDTA as anticoagulant after informed consent was obtained.

Analysis of HbF From Cord Blood and Blood of SS Patients

The composition of the γ-chains of HbF from newborn babies (i.e., the percentages of γ, γ', and γ'' chains) was determined in red cell lysates using the HPLC chromatographic method described before. The HbF of SS blood samples was isolated by DEAE-cellulose chromatography and next analyzed by the same HPLC method.

Analysis of HbF From Blood of HbS Heterozygotes

Since all 33 blood samples had HbF levels below 1% (estimated as % FAD by the method of Betke et al.), a special procedure was developed to isolate this HbF. The initial step concerning macrochromatography on columns of DEAE-cellulose. Usually, the
hemoglobin from 20 ml of blood was applied to two 3.0 × 25 cm columns and the chromatograms developed overnight at room temperature using a slow NaCl gradient (0.005 M in the initial developer and 0.02 M in the limiting developer; the constant volume mixer serving 2 columns had a volume of 1000 ml) and a flow rate of 50-60 ml/hr. The HbF zone was eluted behind the major HbA zone and was grossly contaminated with HbA and HbA 1 (less than 2% of this Hb was of the fetal type). Rechromatography on a smaller (0.9 × 20 cm) DEAE-cellulose column with a similar type of gradient further increased the HbF level in the fetal Hb zone to 2%-10%, while occasionally the HbF level was as high as 30%. Analysis of this material by HPL chromatography allowed the separation and identification of the three types of γ-chain; however, the presence of contaminating zones in the chromatogram often interfered with the quantitative analysis (see Fig. 2, top chromatogram). Thus, an additional purification step was introduced using HPL chromatography on 4 × 100 mm columns of Synchropak AX 300 and a 0.0-0.05 M sodium acetate gradient. As much as 5 mg Hb can be applied to this type of column. A typical chromatogram is illustrated in Fig. 1. Often, the HbF zone was considerably narrower than shown in this figure, but it was readily distinguishable from the HbA-containing zones. The HbF was eluted as a rather narrow zone, and the amount of HbF in the HbA zone and to a lesser extent in the HbA 1 zone was negligible. The advantage of this method is that the column eluent containing HbF can be applied directly to the C 18 Bondapack column of the HPL chromatographic method used for the separation of the three types of γ-chain. The γ-chains of the isolated HbF zone were free of contaminating material (Fig. 2, bottom chromatogram), but the sample still often contained up to 50% HbA as judged from the size of the α-chain zone, which elutes behind the α-chain and well ahead of the γ-chains. The chromatogram shown in Fig. 1 concerns the HbF of an HbS heterozygote who is also heterozygous for the ε γ chain. The data listed in this figure illustrate that the three types of HbF (i.e., with the different types of γ-chains) are equally distributed over the HbF zone.

---

**RESULTS**

The number of SS patients studied was 139. Of these, 97 were older than 6 yr. at which age the delayed switch in the G γ to a γ ratio from newborn to adult values is considered complete. Twenty-two of these 97 SS patients (22.7%) had high G γ values [62.0% + 3.4% (SD)], while the remaining 75 patients had low G γ values (40.8% ± 4.0%). The high number of SS patients with G γ values of about 60% is surprising and quite different from that reported before (12% of the SS patients discussed by Huisman et al. had high values). Additional data confirm the earlier observation that the level of G γ chain in the HbF of the older SS patients is not dependent on the percent HbF present in the blood sample.

Four of the 35 SS newborn babies were ε γ heterozygotes. The level of ε γ chains in the HbF of these 4 babies averaged 69.5%, which is comparable to the values found for 31 SS newborn babies [average 71% ± 2.5% (SD), range 66%-76%] without the ε γ heterozygosity and for normal newborn infants.

**HbS Heterozygotes**

Figure 3 compares the percentages of the Gγ chain in the HbF isolated from blood of 12 HbS heterozygotes before and after further purification by HPLC. None of the “low Gγ chain” producers moved after HPL chromatography into the “high Gγ chain” category. The HPLC rechromatography approach has the advantage that acceptable data could be obtained for samples with low HbF levels as well as for samples in
which the presence of contaminating compounds prevents the quantitation of three types of $\gamma$-chains.

Figure 4 gives the $\delta\gamma$ chain values for 11 SS patients, 18 of their AS parents, and 15 additional HbS heterozygotes. The data from all but 8 HbS heterozygotes were obtained from analyses of HbF samples that had been purified by HPL chromatography. Five of the 11 SS patients had high $\delta\gamma$ chain values, while the $\delta\gamma$ value averaged 41% for the other SS patients. Four of the 18 AS parents had high $\delta\gamma$ chain values but the other 14 had low $\delta\gamma$ values that fell between 24% and 48.5%. Similar percentages were seen for the 15 additional HbS heterozygotes; 2 of these had high values. Two of the 18 AS parents had an additional $\delta\gamma^T$ heterozygosity, while 8 of the highly selected, nonrelated HbS heterozygotes had this anomaly.

Table 1 lists the data for the individual 11 SS patients (arranged according to age into the 2 categories), their 11 mothers, and 7 fathers. Only 4 parents had HbF with high $\delta\gamma$ chain values, 2 in each of the 2 categories of SS patients. None of the 5 SS patients with high $\delta\gamma$ chain values had both parents producing

*All AS parents had HbF$_{AS}$ level below 1%.
†These parents have an $\delta\gamma^T$ heterozygosity.
Table 2. The Levels of the Three γ-Chains in the HbF of Some Parents of SS Newborn Babies With an *γ¹* Heterozygosity

<table>
<thead>
<tr>
<th>SS Newborn</th>
<th>Parent</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ¹</td>
<td>γ¹</td>
</tr>
<tr>
<td>1</td>
<td>65.5</td>
</tr>
<tr>
<td>2</td>
<td>69</td>
</tr>
<tr>
<td>3</td>
<td>69.5</td>
</tr>
<tr>
<td>4</td>
<td>73.5</td>
</tr>
</tbody>
</table>

Table gives some of these data. The relative amount of γ¹ chain in the 4 SS babies was 44% (i.e., the percent γ¹ of total γ). This is comparable to values found in non-SS babies. Similar data were obtained for 24 AS babies (see also Fig. 5) with an average γ¹ value (in percent of total γ) of 45% ± 35% (SD). Previous studies that analyzed the frequency of the γ¹ chain gene in the black populations have indicated that the genotype with the γ¹ mutant in trans to the β mutation occurs 3 times more frequently than that in which it is present in cis. Thus, perhaps 6 of the 24 AS babies had the γ¹ chain gene in cis to the β mutation. The absence of 2 distinct groups with different relative γ¹ values among the AS babies (Fig. 5) suggests an equal expression at birth of the γ¹ chain genes on both chromosomes.

The mothers of three SS babies were γ¹ positive (Table 2) and presumably carry the γ¹ anomaly in cis to the β mutation, although the in cis assignment is only definite for the mother of baby no. 2. The average γ¹ value (54%) was 6 times that of the γ¹ chain (9%). One additional HbS heterozygote with the same condition showed a much higher relative γ¹ chain value (Fig. 5). The 41% was found in the HbF of an AS father of an AS baby who had an γ¹ homozygosity; this sample was used extensively in the development of the new HPLC purification procedure (Fig. 1). Data from samples of 2 AS heterozygotes with an γ¹ chain heterozygosity in trans are also presented; these persons were parents of 2 SS patients who were negative for the γ¹ chain (Table 1). The low values of 9.5% γ¹ (in percent of total γ chain) were similar to those seen in the mothers of the 3 γ¹ positive SS babies.

DISCUSSION

Previous studies concerning the heterogeneity of the HbF from blood of black adult β-thalassemia hetero-

HbF with comparable percentages, but the parents of 3 SS patients with low γ chain values also had HbF with similarly low values.

Analysis of the HbF from AS parents of SS patients with or without an γ¹ heterozygosity offered the opportunity for an initial evaluation of the γ chain synthesis by the γ chain gene in cis or in trans to the β mutation. Table 2 gives some of these data. The relative amount of γ¹ chain in the 4 SS babies was 44% (i.e., the percent γ¹ of total γ), which is comparable to values found in non-SS babies. Similar data were obtained for 24 AS babies (see also Fig. 5) with an average γ¹ value (in percent of total γ) of 45% ± 35% (SD). Previous studies that analyzed the frequency of the γ¹ chain gene in the black populations have indicated that the genotype with the γ¹ mutant in trans to the β mutation occurs 3 times more frequently than that in which it is present in cis. Thus, perhaps 6 of the 24 AS babies had the γ¹ chain gene in cis to the β mutation. The absence of 2 distinct groups with different relative γ¹ values among the AS babies (Fig. 5) suggests an equal expression at birth of the γ¹ chain genes on both chromosomes.

The mothers of three SS babies were γ¹ positive (Table 2) and presumably carry the γ¹ anomaly in cis to the β mutation, although the in cis assignment is only definite for the mother of baby no. 2. The average γ¹ value (54%) was 6 times that of the γ¹ chain (9%). One additional HbS heterozygote with the same condition showed a much higher relative γ¹ chain value (Fig. 5). The 41% was found in the HbF of an AS father of an AS baby who had an γ¹ homozygosity; this sample was used extensively in the development of the new HPLC purification procedure (Fig. 1). Data from samples of 2 AS heterozygotes with an γ¹ chain heterozygosity in trans are also presented; these persons were parents of 2 SS patients who were negative for the γ¹ chain (Table 1). The low values of 9.5% γ¹ (in percent of total γ chain) were similar to those seen in the mothers of the 3 γ¹ positive SS babies.

DISCUSSION

Previous studies concerning the heterogeneity of the HbF from blood of black adult β-thalassemia hetero-

Fig. 5. The relative amounts of the γ¹ chain (as percent of total γ chain) in the HbF of 24 HbS heterozygous babies. 4 adult HbS heterozygotes with the γ¹ chain assumed to be in cis and 2 with the γ¹ chain in trans to the β mutation. Data after the HbF was further purified by HPLC.
zygotes indicated a tremendous variation in the $^6\gamma$ to $^\alpha\gamma$ ratio with $^\gamma\gamma$ values varying between 15.5% and 83.5%.

Two rather distinct categories were observed in these $\beta$-thal heterozygotes with low $^6\gamma$ values (average 32.5%) and with high $^6\gamma$ values (average 64.2%).

The possibility exists that the $\gamma$-chain genes located on the $\beta$-thal chromosome are more active than their counterparts in trans, and indeed, data from extensive family studies involving subjects with an additional $^\alpha\gamma$ $\gamma$-chain heterozygosity have lent support to this suggestion.

A clear distinction into two categories has not been observed for the HbF of normal adults. Perhaps the presence of specific genetic markers, such as the $\beta^4$ and $^\alpha\gamma$ mutations located either on the same chromosome or on opposite chromosomes, may be helpful in evaluating the contribution by the various $\gamma$-chain genes to the $\gamma$-chain composition of HbF. Unfortunately, the presently available data for adult HbS heterozygotes with an $^\gamma\gamma$ $\gamma$-chain heterozygosity either in cis or in trans to the $\beta^4$ mutation have not contributed to any great extent. It appears that while at birth the $\gamma$-chain genes on both chromosomes are contributing equally to the levels of the different types of $\gamma$-chain (Table 2; Fig. 5), the synthesis of the $\gamma$-chains in the small amount of HbF (FAD < 1%) in adult HbS heterozygotes may be directed in unequal quantities by genes in cis or in trans to the $\beta^4$ mutation, or by both sets of genes.

One wonders if this entire situation is much more complicated, for instance, by assuming that the HbF-containing cells ("F-cells") of the normal adult do not all have the same $^6\gamma$ to $^\alpha\gamma$ ratio. Support for this possibility comes from earlier data indicating that reticuloocyte from patients with various types of $^6\gamma\gamma$-HPFH synthesize an HbF with mainly $^6\gamma$ $\gamma$-chains. A similar type of heterogeneity might exist among the "F-cells" of the normal adult, the adult HbS heterozygote, and the adult SS patient. Thus, the observed variability in $^6\gamma$ chain values of the isolated HbF might reflect a cellular heterogeneity. A recessive inheritance of a modification in the regulatory mechanism would explain most data, except for the observations made in families with SS patients who have HbF with either low $^6\gamma$ values (13 families with 2 SS children; one family each with 3 and 4 SS children) or high $^6\gamma$ values (3 families with 2 SS children; one family each with 3 and 4 children).

The exact cause of the high $^6\gamma$ chain level in the HbF of over 20% of the SS patients remains unexplained. Although the rather limited family data do not support a simple inheritance pattern (Table 1), a familial basis for this anomaly has repeatedly been demonstrated. Longitudinal studies in some newborn SS babies have suggested the support that high or low $^6\gamma$ chain levels persist over a long period of time and that in some individuals the second switch after birth, namely that of the "newborn" $^6\gamma$ to $^\alpha\gamma$ value of 7:3 to the "adult" value of 4:6, may not take place. The use of high pressure liquid chromatography as the final purification step in the isolation of HbF is of value because this method enhances the possible evaluation of HbF from blood samples with low levels (<1%) of this fetal protein. The elimination of unknown compounds that elute at positions in the chromatogram occupied by the $\gamma$-chains makes the quantitative aspect of the Bondapack C18 HPLC procedure more reliable.

ACKNOWLEDGMENT

The authors are indebted to B. Joseph, B. L. Abraham, H. F. Harris, and L. Walker for their help in the family studies. Drs. C. Altay and P. F. Milner supplied blood samples from some of the SS patients.

REFERENCES

3. Efremov GD, Wilson JB, Huisman THJ: The chemical heterogeneity of human haemoglobin F: Direct evidence for the existence of three types of $\gamma$ chains, the $^6\gamma$, $^4\gamma$, and $^\alpha\gamma$ chains. Biochem Biophys Acta 579:421, 1979
10. Huisman THJ, Webber B: The frequency of the $^\gamma$ $\gamma$ gene in
the presence and absence of the $\beta^+$ or $\beta^-$ gene in the black population of the southeastern USA. Hemoglobin 5:441, 1981


