Elevated HbF Associated With an Unstable Hemoglobin, Hemoglobin Saint Etienne: Hb Synthesis in Blood BFUe in Culture

By U. Testa, Y. Beuzard, W. Vainchenker, M. Goossens, A. Dubart, N. Monplaisir, C. P. Brizard, Th. Papayannopoulou, and J. Rosa

The red blood cells of a patient heterozygous for the unstable Hb Saint Etienne (p92 His -> Gin) contained 19% of fetal hemoglobin (Hb). Study of his family suggested that the proband had inherited the Swiss type of hereditary persistence of fetal hemoglobin (HPFH) from his father who had 1.2% of HbF and 8% of F cells. In order to gain further insight into the mechanism of the elevated production of HbF in the proband, hemoglobin synthesis was studied in 14-day cultures of blood erythroid burst-forming units (BFUe). The culture of cells from the proband and from his father resulted in an average production of 30% HbF, while HbF synthesis in the mother’s BFUe was only 12%. These findings suggest that individuals with the Swiss HPFH may have a proportion of circulating precursors that possesses a high potential for HbF synthesis in vitro as compared to normal controls. The elevated production in vivo of HbF in the proband can therefore be regarded as an expression of this high potential for HbF synthesis, which occurs under the influence of an increased erythropoietic stimulation induced by the unstable hemoglobin. Study of single erythroid bursts derived from the proband’s blood cells showed a reciprocal relationship between synthesis of HbF and of Hb Saint Etienne, while the amount of HbA remained constant. This result suggests that the increased synthesis of HbF seen in the propositus is linked to one chromosome only, i.e., that which bears the Saint Etienne mutation.

The heterocellular hereditary persistence of fetal Hb (HPFH) is characterized by an increased level of HbF, which is heterogeneously distributed among the red blood cells (RBC) of otherwise hematologically normal adults. The heterozygous state of the Swiss type of HPFH corresponds to a slight increase of HbF (1%–3%) and of the HbF-containing cells (F cells > 6%). The Swiss HPFH has been found in association with sickle cell anemia, in which case it gives rise to a much higher level of HbF and of F cells.

This article describes the association of the Swiss type of HPFH with a chronic hemolysis induced by an unstable Hb, Hb Saint Etienne (HbSE). The previous study of the proband indicated the following Hb levels in his blood: HbF (19%), HbA (52%), HbSE (26%) and HbA2 (3%). His parents, as confirmed by the study of genetic markers, were found to be hematologically normal, and the high level of HbF of the proband could not be explained initially. The present reevaluation of this family, with special reference to their F cells, indicates that the father of the

From the Unité de Recherches sur les Anémies, INSERM U.91, Hôpital Henri Mondor, Creteil; Centre Départemental de Transfusion Sanguine, C.H.U., Saint Etienne; and the University of Washington, Seattle, Wash.

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Address reprint requests to Yves Beuzard, Unité de Recherches sur les Anémies, INSERM U.91, Hôpital Henri Mondor, 94010 Creteil, France.

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proband possesses the criteria anticipated for the Swiss type of HPFH (i.e., HbF 1.2%, F cells 8%). The sister of the proband had borderline level HbF, 1%, and F cells, 7%. In addition, the culture of blood BFUe from the propositus and from his parents provides new information on the cellular mechanism of expression of the γ gene in heterocellular HPFH in vitro and on its amplification in vivo upon an increased erythropoietic stimulation.

MATERIALS AND METHODS

Hematologic indices and the survival of 51Cr-labeled RBC were determined according to standard methods.

Culture Procedure

Twenty milliliters of blood were collected and mixed with 200 units of preservative-free heparin. The mononuclear cells were isolated by centrifugation on a cushion of Ficoll metrizoate.9 The cells were washed three times in a medium (Eurobio, Paris, France), and the plasma clot cultures were established in 35-mm Falcon Petri dishes containing 10^6 nucleated cells/ml.10S Slight modifications were used: a medium replaced the NCTC 109 medium, human AB serum was used instead of fetal calf serum, and CaCl2 was added at a final concentration of 6mM.12 Beef embryo extract was omitted. Erythropoietin from sheep plasma (anemic sheep plasma erythropoietin step III preparation, Connaught Research Laboratories, Toronto, Canada) was added at zero time at a concentration of 1 or 3 units/ml. At day 13, 100 μCi of 3H-leucine (50 Ci/mM Commissariat à l'Energie Atomique, Saclay, France) was added in 0.3 ml of NCTC 109 medium made leucine-free (Eurobio, Paris, France). The cells were harvested at day 14. Digestion of the clot by pronase (1 mg/ml, Calbiochem, San Diego, Calif.) for 10 min at room temperature allowed the recovery of all the cells from one dish. Individual bursts were removed after partial digestion13 using an inverted microscope at x40 magnification. Red blood cells were added as carrier when necessary. The cells were washed extensively with Hanks medium and then with sodium chloride (0.15 M). The cells were frozen at -80°C until use.

Hemoglobin Study

The blood HbF level was determined according to the method of Betke.14H Hemoglobin fractions were estimated by starch block electrophoresis8 because other methods did not separate Hb Saint Etienne from HbA2. The percentage of HbF-containing cells was estimated by immunofluorescence, as described previously.17 Hemoglobin synthesis was evaluated on reticulocytes from the blood used for culture and also on BFUe. The reticulocytes were incubated as outlined earlier. The white cells and platelets were removed after incubation by filtration on cellulose. The recovery of the radioactivity incorporated into the proteins of reticulocytes was close to 90%.

The radioactivity incorporated into hemoglobins was estimated by two methods. The first method used was the separation of the globin chains by chromatography on carboxymethyl cellulose in urea.19 Microcolumns (0.4 x 20 cm) gave suitable optical density profiles with as little as 0.2 mg of globin using the Isco type 6 optical unit and the Isco 1133 recorder (Lincon, Nebr.). The carboxymethyl cellulose Whatman CM 52 was first sedimented in water and then extensively equilibrated with sodium phosphate buffer (0.035 M) at pH 6.70 until the resistance of the resin supernatant was identical to that of the buffer. The ultrograd gradient (LK8, Bromma, Sweden) was used to improve the separation of the γ and β globin chains. The buffer described above was used as first buffer in the gradient. The pH and concentration of the second phosphate buffer used were 6.70 and 0.035 M, respectively. Fractions (1.2 ml) were collected at a flow rate of 8 ml/hr for 16 hr. An aliquot (1 ml of each fraction) was used for the determination of radioactivity after mixing with 10 ml of Lumagel (Lumac, Belgium). This method was suitable for the study of HbF synthesis as estimated by radioactivity of the γ/α ratio of globin chains and for the determination of the balance of the synthesis of the α and non-α globin chains. However, it was not possible to separate the β from the β Saint Etienne globin chains (Fig. 1). In addition, this technique, even after miniaturization, is not sensitive enough for the study of the hemoglobin synthesis in a single colony.

Consequently, a second method, electrophoresis on cellulose acetate strips (4 cm x 17 cm; Chemetron, Milano, Italy), was used. This technique allowed the separation of HbA, HbF, and HbSE.8
Case Findings

Clinical, hematologic, and biochemical studies of the Saint Etienne case have been previously reported.\textsuperscript{22,23} The proband, a boy of French origin, was 6-yr-old during the initial study; he is now 13-yr-old. The hemolytic process did not impair his growth, exercise, or learning. He always had a slightly enlarged spleen. The usual indices under steady-state conditions were as follows: RBC, 4,180,000/cu mm; hematocrit, 43.4; Hb, 11.3/dl; MCV, 104; MCH, 31.5; MCHC, 30.5; reticulocytes, 5%; unconjugated bilirubin, 12 mg/liter; and haptoglobin, <0.2 mg/liter. The half-life of the $^{51}$Cr-labeled red cells was 6.5 days. The hemolytic process was increased from time to time as judged by the enlarged spleen and hematologic indices. His hemoglobin pattern also showed variations: i.e., HbA changed from 48% to 55%, HbF varied from 12% to 20%, and Hb Saint Etienne
Fig. 2. Electrophoretic pattern on cellulose acetate (pH 8.7) given by the hemolysate from the proband; amido black staining. (B) After the addition of haptoglobin. A, HbA; F, HbF; SE, Hb Saint Etienne; A₂, HbA₂; AC, carbonic anhydrase; Hp, haptoglobin; Hp-Hb, haptoglobin-hemoglobin complexes.

Fig. 3. Radioactivity pattern obtained upon electrophoresis of the hemoglobin of the proband’s BFUe on cellulose acetate strip in the absence of haptoglobin (---) and in the presence of haptoglobin (------). The dotted area corresponds to the difference between the two curves.
from 22% to 35%. Hemoglobin F was heterogeneously distributed among the red blood cells as determined by the technique of Kleihauer. Study of hemoglobin synthesis in reticulocytes showed a balanced synthesis of α and non-α globin chains. Fractionation of the red blood cells according to their little-i antigen content was performed by agglutination with a human anti-little-i antibody. The population rich in little-i antigen had a higher level of Hb Saint Etienne (34%) and a lower level of HbF (14%) than the unfractionated population. The inverse was obtained with the population poor in little-i antigen, i.e., HbSE, 19% and HbF, 25%. On the basis of the radioactivity incorporated into the hemoglobin fractions and the i-antigen level of the reticulocytes, the heterogeneity of the cell population appeared to be independent, at least partially, of the RBC selection in the peripheral blood.

The parents and the only sister of the proband were normal on clinical and hematologic examination. Their hemoglobin exhibited a normal electrophoretic pattern and contained normal amounts of HbA2. The parents' cells displayed a balanced synthesis of globin chains. The study of the genetic markers confirmed the filiation.

F-Cells Study

Table 1 summarizes the proportion of F cells and HbF in the proband and his family. The proband had a higher proportion of HbF and F cells than normal controls. The percentage of F cells was smaller in the reticulocyte fraction than in the total RBC population, thus indicating the greater survival of F cells. However, selection of F cells could not account alone for the high HbF level, since 25% of the reticulocytes contained HbF. The father exhibited a slight increase of both HbF (1.2%) and F cells (8%) as compared to the normal values (HbF < 1%, F cells 0.5%-7%). The mother showed normal values. These results indicated that the high level of HbF and F cells in the propositus was most probably inherited from his father, but that the HbF was not expressed to the same extent in vivo in the father and his son. The sister of the propositus had a borderline level of HbF (1.0%) and F cells (7%).

Hemoglobin Synthesis

Data obtained from studies of synthesis of hemoglobins in reticulocytes and in blood BFUe are summarized in Table 2. The electrophoresis of the hemoglobins

<table>
<thead>
<tr>
<th>Percent of HbF</th>
<th>Percent of F Cells</th>
</tr>
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<tbody>
<tr>
<td>Propositus</td>
<td>12.02 ‡</td>
</tr>
<tr>
<td>Mother</td>
<td>0.33</td>
</tr>
<tr>
<td>Father</td>
<td>1.30</td>
</tr>
<tr>
<td>Sister</td>
<td>1.0</td>
</tr>
<tr>
<td>Normal controls</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

* Determined by alkali denaturation.
† Determined by immunofluorescence.
‡ Total RBC population.
§ Reticulocytes.
Table 2. Comparison of HbF Synthesis in Reticulocytes In Vitro and In BFUe Determined by: Electrophoresis of the Hemoglobins and by Separation of the Globin Chains on CMC-Cellulose (see Parentheses)

<table>
<thead>
<tr>
<th></th>
<th>Reticulocytes</th>
<th>Culture of BFUe</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propositus</td>
<td>15 (19)</td>
<td>27 (35)</td>
</tr>
<tr>
<td>Mother</td>
<td>&lt;2 (&lt;2)</td>
<td>12</td>
</tr>
<tr>
<td>Father</td>
<td>&lt;2 (&lt;2)</td>
<td>29 (30)</td>
</tr>
<tr>
<td>Normal controls</td>
<td>&lt;2 (&lt;2)</td>
<td>10-20 (8.5-19.5)</td>
</tr>
</tbody>
</table>

(Fig. 2) and the separation of the globin chains by chromatography (Fig. 3) indicated similar proportions of HbF and of γ-chain synthesis. Both methods were valid for the study of hemoglobin synthesis in reticulocytes derived from fresh blood or in cultures of blood BFUe. The proband reticulocytes synthesized HbF in a proportion close to the HbF level estimated by the chemical method of Betke in the total RBC population. This result confirmed that the preferential survival of F cells was not the main factor accounting for the high level of HbF in the proband. Hemoglobin F synthesis in the bursts was higher than that seen in vivo, but the amplification was small compared to that of the bursts of the father. In addition, the father had a low level of HbF synthesis in his reticulocytes (<2%) in contrast to the high level of HbF synthesized by his bursts (30%), which was similar to that of the propositus's bursts.

The rate of HbF synthesis in the mother's reticulocytes and blood BFUe was in the normal range in both cases. Eleven normal controls had levels of HbF synthesis in culture of their blood BFUe that ranged from 8.5% to 19.5% with a mean of 14.5% (results not shown).

Twelve colonies from one culture dish of the propositus's BFUe were individually recovered by careful pipetting under an inverted microscope. Ten were suitable for analysis of the radioactivity in the hemoglobin fractions by cellulose acetate electrophoresis; two did not incorporate sufficient radioactivity to be analyzed with accuracy. The proportion of the radioactivity incorporated into the hemoglobin fractions of 10 colonies is shown in Table 3. Hemoglobin A synthesis remained nearly constant (46%-50%) in all the colonies studied except for colonies 2 (53.5%) and 10 (43%). Such small variations may be due to methodology inaccuracy. In contrast, the synthesis of hemoglobins F and SE varied widely at the expense of each other; the inverse correlation between them (p < 0.0001) is shown in Fig. 4. This result indicated that hemoglobins A, SE, and F were expressed in all the colonies examined. Unfortunately, in view of the small number of colonies tested, we could not exclude the existence of colonies that synthesized only HbF or adult

Table 3. Percentage of Synthesis of Hemoglobins St. Etienne, A, and F in BFUe From the Propositus

<table>
<thead>
<tr>
<th>Colony</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>% HbSE</td>
<td>35</td>
<td>24</td>
<td>42</td>
<td>32</td>
<td>36</td>
<td>40</td>
<td>40</td>
<td>44</td>
<td>34</td>
<td>45</td>
</tr>
<tr>
<td>% HbF</td>
<td>16</td>
<td>22.5</td>
<td>11</td>
<td>18</td>
<td>15</td>
<td>12</td>
<td>13</td>
<td>10</td>
<td>20</td>
<td>12</td>
</tr>
<tr>
<td>% HbA</td>
<td>49</td>
<td>53.5</td>
<td>47</td>
<td>50</td>
<td>49</td>
<td>48</td>
<td>47</td>
<td>46</td>
<td>46</td>
<td>43</td>
</tr>
</tbody>
</table>
DISCUSSION

Levels of HbF above 10% have been described in association with only 2 unstable hemoglobins: Hb Sabine β92 Leu → Pro and Hb Saint Etienne. Lower but still abnormal levels of HbF have been reported in conjunction association with some other unstable Hb variants. The unstable Hb Istanbul, which has the same amino acid substitution as HbSE, did not occur in combination with an increased level of HbF. Consequently, it is most unlikely that the high level of HbF present together with HbSE is due to mutation or to the unstable hemoglobin per se.

The high level of HbF in the propositus most probably results from two different mechanisms. One genetically determined, i.e., the Swiss type of HPFH, and the other being the permanent stimulation of erythropoiesis. HbSE may increase erythropoiesis on two accounts. HbSE is a semihemoglobin lacking heme on its β chain and, as a consequence, has a lower capacity for oxygen transport. It also has a high oxygen affinity. In addition, the instability of HbSE gives rise to an increased hemolysis.

The main argument for the existence of the Swiss type of HPFH is the discovery in the father and sister of a slight increase in the proportions of HbF and F cells in the absence of any hematologic or clinical abnormality or imbalance in globin chain synthesis. In the propositus, the high level of HbF is heterogeneously distributed, and there is no imbalance in globin chain synthesis. The mother of the proband has normal proportions of HbF and F cells. It is known that the heterocellular form of HPFH is inherited as an autosomal and dominant character. The association of sickle cell anemia with heterocellular HPFH increases the proportion of HbF to about 20%, a level much higher than that induced either by HPFH or by sickle cell anemia alone. The association of heterocellular HPFH and of β-thalassemia can also result in high levels of F cells and of HbF.

The variation from 12%–20% in the proband's HbF in vivo can be explained by periods of increased hemolysis, during which the cells containing the unstable Hb are removed at a faster rate, thereby increasing the proportion of F cells, which
have a longer survival time. A similar mechanism has been proposed in order to explain the fluctuation of HbF seen in patients with sickle cell disease. The lower percentage of F cells (25%) in the reticulocyte fraction as compared to the total RBC population in the proband confirms the suggestion that the F cells of the proband have a longer survival time than the other RBC. However, since a high proportion of reticulocytes contain HbF, another mechanism appears necessary to account for the production of a greater number of F cells than that occurring in normal controls. A stimulated erythropoiesis may produce this effect when associated with a HPFH of the Swiss type. It would be of interest to study the American family in which there is an association of the unstable Hb Sabine with 12% F in order to determine whether a heterocellular HPFH is also present. Another case of Hb Sabine has been found in Sardinia where the HbF level was 3% (A. Cao, L. Bernini, R. Galanello, unpublished results).

The culture of blood BFUe in this study has provided new information on the expression of HbF. While the values for HbF synthesis found in the mother was within the normal range (12%), the HbF synthesized by bursts from the peripheral blood of the father, a carrier of the Swiss HPFH, was significantly higher (30% HbF). Moreover, this value was similar to that found in the BFUe from the proband (35%). In vivo, the level of HbF and F cells was quite different in the proband and his father. To explain this discrepancy, it could be hypothesized that the potential for HbF synthesis is similar in the proband and his father, as shown by the results of BFUe culture in which the conditions of stimulation were identical and maximal. However, the difference between the stimulation of erythropoiesis in vivo (normal in the father, high in the propositus) could explain the observed differences in HbF synthesis. In addition, the relatively small twofold increase in the synthesis of HbF, observed in vitro in the propositus, could indicate that the stimulation of his erythropoiesis approaches the maximum in vivo. We suggest that the mutation of the Swiss HPFH gene increases the proportion of erythroid stem cells that are able to synthesize fetal hemoglobin, a proportion normally fixed at the end of the ontogenic switch of hemoglobins. This capability of HbF synthesis by BFUe would be only slightly expressed in vivo under normal erythropoietic conditions but would be amplified in vitro. The tremendous increase in HbF synthesis in vitro by the bursts of the father supports this hypothesis. An alternative hypothesis is that the mutation responsible for HPFH enhances the sensitivity of the BFUe cells, which differentiate under a stimulation (still unknown) toward more HbF synthesis than in the absence of the HPFH mutation.

The cells of normal adults also have a peculiar behavior with regard to HbF synthesis in culture, and this deserves discussion. A high stimulation of HbF synthesis in cultures of blood BFUe (10–20-fold) as compared to the expression of HbF in vivo in normal individuals has been reported. Seven normal adults have been studied in our laboratory and Hb synthesis in the reticulocytes from their fresh blood was undetectable. The proportion of F cells and of HbF in peripheral blood was in the normal range. Hemoglobin F synthesis in blood BFUe at 14 days of culture ranged from 8.5% to 19.5% with a mean of 14.5% (results not shown), which is in agreement with the results of the other investigations. In order to explain this activation of HbF synthesis in cultures of blood BFUe, it has been suggested that early erythroid precursors have a greater ability to express γ genes.
than more differentiated precursors and that the recruitment of these cells in culture is responsible for the consistent activation of fetal hemoglobin synthesis.\textsuperscript{34,35} Thus culture may amplify the capability of HbF expression and may therefore be able to reveal differences among the normal population. The level of HbF synthesis in blood BFUe culture remained identical when the study of one normal adult was performed on three different occasions (results not shown). The level of HbF synthesis in blood BFUe may therefore be a characteristic of one person; it would be of interest to determine if it is genetically determined.

The inverse correlation of HbF and HbSE synthesis in individual burst colonies resembles the results obtained with two populations of reticulocytes.\textsuperscript{8} Concordant results obtained both with BFUe and reticulocytes indicated that HbSE synthesis was increased when HbF synthesis was decreased (regulation in cis). Consequently, HbF synthesis, at least in the bursts studied, was linked to one adult hemoglobin gene, the $\beta$ SE gene. Such linkage is in good agreement with the genetic studies of the Swiss type of HPFH found in association with sickle cell disease and with thalassemia.\textsuperscript{3,6,7} These studies indicated that a heterocellular HPFH gene was inherited as an autosomal and dominant character. It is linked in cis to the $\beta$ SE gene. It was found in cis to the $\beta^s$ gene. Ten percent of recombinants were found in 121 offspring.\textsuperscript{6} The present study provides arguments that the new mutation of the $\beta$ gene leading to the HbSE mutation occurred on the chromosome bearing the HPFH. The unstable Hb represents a marker of this chromosome and by increasing erythropoiesis amplified the expression of the HPFH gene.

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Hb SYNTHESIS IN CULTURE

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