F-Cells in the Adult: Normal Values and Levels in Individuals With Hereditary and Acquired Elevations of Hb F

By W. G. Wood, G. Stamatoyannopoulos, G. Lim, and P. E. Nute

Specific antibodies to human fetal hemoglobin were prepared and, after conjugation with a fluorescent dye, were used to determine the distribution of Hb F-containing cells in blood smears from normal adults and individuals with hereditary and acquired conditions associated with abnormal levels of Hb F. The mean proportion of F-cells in normal persons was 2.7% ± 1.4%, with a range of 0.5%–7.0%. Proportions of F-cells were increased in persons with several acquired and inherited disorders that are associated with an increased percentage of Hb F in hemolysates. A strong linear correlation between the amount of Hb F and proportion of F-cells was observed. This technique may prove useful in studies of a variety of disorders associated with Hb F elevations and also in investigations of the mechanisms controlling the transition from fetal to adult hemoglobin during the course of human development.

CYTOCHEMICAL METHODS for demonstrating the presence of fetal hemoglobin (Hb F) in individual red cells have been usefully applied to the study of several hematologic disorders. Thus, the acid-elution procedure developed by Kleihauer et al. has been instrumental in the delineation of the various forms of hereditary persistence of fetal hemoglobin (HPFH), in making the distinction between F-thalassemia and HPFH, and in the differential diagnosis of sickle cell syndromes associated with marked Hb F elevations. Furthermore, the Kleihauer test has been successfully used in the diagnosis of fetal-maternal transfusion and in the development of procedures for the immunologic prevention of hemolytic disease of the newborn arising from Rh incompatibility.

Immunologic techniques provide an alternative to physicochemical methods for the demonstration of Hb F in single erythrocytes. The ability of antibodies conjugated with fluorescent compounds to bind to Hb F-containing cells (F-cells) has been demonstrated by Tomoda and Hosoi, and an elegant double-labeling technique for the detection of both hemoglobins A and F in single cells has been described by Dan and Hagiwara. In contrast to the acid-elution procedure, which can generate artifacts, immunologic methods for assessing the distribution of Hb F among red cells can be made very sensitive and highly specific. In this report, we describe the development of purified anti-Hb F antibodies and their use in assessing the distribution of F-cells in normal individuals as well as in persons with various inherited and acquired disorders characterized by abnormal levels of Hb F. Our data show that this method...
measures the distribution of small amounts of Hb F in red cells with greater sensitivity than other techniques currently in use.

MATERIALS AND METHODS

Production of Antibodies

Purification of Hb F. Pure Hb F was obtained from normal cord bloods by subjecting hemolysates to chromatography on DEAE-Sephadex columns to remove traces of the minor derivatives of Hb A. The pure Hb F (as judged by starch-gel electrophoresis) was converted to cyanmethemoglobin by dialysis against Drabkin's solution, after which excess cyanide was removed by dialysis against phosphate-buffered saline, pH 7.0.

Immunization schedules. Published schedules used in raising antihemoglobin antibodies vary in the total amount of antigen employed, the species of animal immunized, and the period of time over which the antigen is administered. Four separate immunization schedules were compared using New Zealand white rabbits (purchased from different animal dealers) to assess the efficacy of different amounts of antigen administered over various periods of time. These schedules, summarized in Table 1, cover a wide range of antigen doses, including the most and the least intensive of those reported. Serums were tested 7 days after the final injection, and the maximum dilution of antiserum producing a visible precipitate when reacted with 0.15 mg/ml Hb F on Ouchterlony double-diffusion plates was taken as a rough measure of antibody activity (Table 1). In general, the variation of response between animals on the same schedule was at least as great as variation between schedules; hence, the large amounts of antigen administered according to the most intensive schedule were no more effective in stimulating antibody production than were those of the schedules using less antigen and fewer injections. Therefore, schedule II was adopted for the majority of immunizations. Responding animals were bled of 50 ml weekly, and booster doses of 10 mg of antigen in Freund's incomplete adjuvant were administered as soon as a drop in antibody titer was noted (approximately every 3 wk). All antisera reactive against Hb F were also reactive against hemoglobins A and A2 (Fig. 1A) and therefore required purification.

Table 1. Immunization Schedules Used in the Production of Anti-F Antibodies

<table>
<thead>
<tr>
<th>Schedule</th>
<th>Injection Days</th>
<th>mg of Hb F injected</th>
<th>Adjuvant*</th>
<th>Injection Sites</th>
<th>Maximum Serum Dilution Reactive With 0.15 mg/ml Hb F</th>
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<tbody>
<tr>
<td>I</td>
<td>0</td>
<td>2</td>
<td>FC*</td>
<td>Subcutaneous</td>
<td>1/2–1/8</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>20</td>
<td>FI*</td>
<td>Subcutaneous</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>0</td>
<td>2</td>
<td>FC</td>
<td>Subcutaneous</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>2</td>
<td>FI</td>
<td>Subcutaneous</td>
<td></td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>10</td>
<td>FI</td>
<td>Subcutaneous</td>
<td>1/4–1/32</td>
</tr>
<tr>
<td>III</td>
<td>0</td>
<td>5</td>
<td>FC</td>
<td>Subcutaneous</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>5</td>
<td>FI</td>
<td>I.M.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>5</td>
<td>FI</td>
<td>I.M.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>5</td>
<td>FI</td>
<td>I.M.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>35</td>
<td>5</td>
<td>FI</td>
<td>I.M.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>30</td>
<td>FI</td>
<td>I.M.</td>
<td>1/2–1/16</td>
</tr>
<tr>
<td>IV</td>
<td>0</td>
<td>20</td>
<td>FC</td>
<td>Subcutaneous</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>10</td>
<td>FI</td>
<td>I.M.</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Twice a week</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4 1/2 wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>57</td>
<td>100</td>
<td>None</td>
<td>I. P.</td>
<td></td>
</tr>
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<td></td>
<td>58</td>
<td>10</td>
<td>None</td>
<td>I. V.</td>
<td></td>
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<tr>
<td></td>
<td>59</td>
<td>10</td>
<td>None</td>
<td>I. V.</td>
<td>1/8–1/32</td>
</tr>
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</table>

*FC, Freund's complete adjuvant; FI, Freund's incomplete adjuvant.
Fig. 1. Ouchterlony double-diffusion plates showing the reactivity of the anti-Hb F antiserum (A) before purification, (B) after binding to Sepharose-Hb F and further purification by CM-cellulose chromatography.

Purification of Antibodies

Hemoglobins were covalently bound to Sepharose previously activated with cyanogen bromide. The Sepharose-Hb complexes were washed thoroughly with 0.2 M borate buffer, pH 8.0, containing 1% (w/v) glycine, to saturate the remaining active sites. Weakly bound hemoglobin molecules were eluted by washing of the Sepharose-Hb column with CO₂-water, pH 4.0-4.5. When the eluate was free of hemoglobin, the Sepharose-Hb F was reequilibrated to pH 8.0 with the 0.2 M borate buffer.

The procedure used for antibody purification was a modification of that described by Boyer et al. Antisera were first exposed to Sepharose-bound Hb A (and its minor derivatives) and Hb A₂ to remove antibodies that react with these hemoglobins. The anti-Hb F antibodies remaining in solution were then bound to a column of Sepharose-Hb F. Unbound serum proteins were removed by extensive flushing of the column with borate buffer, pH 8.0; the antibodies were then eluted with CO₂-water, pH 4.0. Small amounts of hemoglobin contaminating the antibody-containing fractions were removed by rechromatography of these fractions using a CM-cellulose (Whatman CM-52) column equilibrated with CO₂-water and developed with 0.05 M NaHCO₃, pH 8.1. The pure anti-F antibodies recovered from one column (Fig. 1B) were dialyzed against distilled water, lyophilized, and stored at -20°C.

Conjugation of Antibodies With FITC

Anti-Hb F antibodies, in amounts ranging from 5 to 10 mg, were conjugated with fluorescein isothiocyanate (FITC, Nutritional Biochemicals Corp.) as described by Nairn. Separation of the antibody-FITC conjugates from excess FITC was achieved by gel filtration in a column of Sephadex G-25, equilibrated and developed with phosphate-buffered saline, pH 7.0.

Application of FITC Antibodies to Blood Smears

Peripheral blood smears were allowed to air-dry overnight, after which they were fixed by immersion in a 9:1 (v/v) acetone: methanol mixture for 5 min. The slides were rinsed with phosphate-buffered saline, pH 7.0, and distilled water, and allowed to surface-dry before the application of 1–2 μl of the solution of FITC-labeled antibodies. The slides were then incubated in a moist chamber for 30–60 min at 37°C, and the unbound antibodies were removed by thorough washing in phosphate-buffered saline followed by rinsing in distilled water.

F-Cell Measurements

Smears were prepared using freshly drawn blood or blood that had been stored at 4°C for periods of up to 10 days. A minimum of 2000 cells were counted on each smear; fields were selected under white light, and the total number of cells was determined. The fields were then examined.
under ultraviolet light, and the fluorescent cells were counted. In this way, bias in selecting fields was avoided, a necessity when relatively low proportions of F-cells are present in the preparation. All F-cell counts were made by the same person, and counts were completed prior to measurement of the proportion of Hb F in the bloods examined.

Quantitation of Hb F in Lysates

The proportions of Hb F in individual lysates were determined using the alkali-denaturation procedure of Betke et al.24

Individuals Examined

Levels of F-cells were measured in blood samples obtained from 140 persons, 82 blacks and 58 whites, who were judged to be hematologically normal during the course of a screening program for hemoglobinopathies. Levels of F-cells were also measured in blood samples from persons with various types of hereditary persistence of fetal hemoglobin, β-thalassemia, sickle cell anemia, and hereditary aplastic anemias. Levels of Hb F and F-cell frequencies were also determined in blood samples from several individuals with acquired hematologic disorders (primarily leukemias).

RESULTS

Specificity of the Anti-Hb F Antibodies

The use of immunofluorescence for the detection of a hemoglobin within erythrocytes requires the production of highly specific antibodies; such antibodies must not cross react with any other hemoglobin present in the same cells. Evidence that the antibodies used in our experiments are specific for Hb F is threefold.

(1) The absence of cross-reactivity with Hb A and its derivatives and Hb A2 on Ouchterlony double-diffusion plates (Fig. 1); however, this observation alone does not exclude the possibility that our preparation might contain non-precipitating antibodies that cross react with Hbs A and A2.

(2) All red cells from persons heterozygous for HPFH fluoresced after exposure to FITC-conjugated antibodies, whereas various proportions of fluorescent cells were observed in disorders known to result in heterogeneous distributions of Hb F-containing cells, e.g., β- and βδ-thalassemias and sickle cell anemia. Moreover, low proportions of F-cells were found in normal individuals. These results were consistent with those obtained by the acid-elution technique of Kleihauer et al.1

(3) With levels of Hb F between 1% and 3%, there is a strong correlation between the proportions of F-cells in smears and the amounts of Hb F in the corresponding hemolysates. The levels of F-cells and proportions of Hb F in 97 subjects are compared in Fig. 2. The correlation of levels of Hb F below 1% with proportions of F-cells is lower ($r = 0.43$) than that calculated for the entire series ($r = 0.93$). Presumably, this discrepancy reflects the relative insensitivity of the alkali-denaturation test when low levels of Hb F are measured, since even pure Hb A gives values for alkali-resistant hemoglobin of 0.3%–0.6%, depending on the hemoglobin concentration.25

The Effect of Storage and Various Anticoagulants on F-cell Counts

To assess the effects of different anticoagulants and time of storage upon F-cell counts, blood samples from a normal adult (Hb F by alkali denaturation = 0.46%) were collected in tubes containing either EDTA, ACD, or
heparin. Slides were prepared immediately and treated with the fluorescent anti-Hb F antibody on the following day. The F-cell counts revealed no significant differences (2.32% in EDTA, 2.21% in ACD, and 2.30% in heparin). The blood collected in EDTA was further tested at intervals of 3–4 days, and all three samples were retested after 15 days. The proportion of F-cells in each sample remained constant throughout this period (Fig. 3).

F-Cell Frequencies in Normal Individuals

Application of FITC-labeled anti-Hb F antibodies to the blood smears of 82 normal black and 58 normal white adults produced the F-cell proportions shown in Fig. 4; a representative smear appears in Fig. 5.

The proportion of F-cells among black individuals ranged from 0.5% to 7.0% with a mean and standard deviation of 2.8% ± 1.6%. In two individuals, the levels of F-cells were well above the normal range, one having 10.2% and the other 14.9% F-cells. The upper limit of the F-cell distribution in whites was difficult to set, as four values were scattered between 6.0% and 9.0%. Values for the mean and standard deviation excluding and including these samples are 2.6% ± 1.0% and 2.9% ± 1.6%, respectively. One subject had over 10% F-cells. The range of normal values reported here was somewhat greater than the range of 0.2%–3.0% reported by Boyer et al. Such discrepancies between the F-cell proportions determined in different laboratories may reflect differences in technique or in the activities of antibody preparations.
**F-cell Frequencies in Inherited and Acquired Hematologic Disorders**

The proportions of F-cells observed in several inherited and acquired hematologic disorders are presented in Fig. 6. These data represent preliminary results obtained from examination of unselected patients, but they do lend support to some generalizations.

1. **Hereditary persistence of fetal hemoglobin (HPFH):** All red cells from heterozygotes for the African variety of HPFH took up the fluorescent anti-
Fig. 5. Peripheral blood smears stained with FITC-labeled anti-hemoglobin F antibody. (A) Normal individual (Hb F, 0.46%; F-cell level, 2.3%). (B) Heterozygote for hereditary persistence of fetal hemoglobin (Hb F, 15.5%; F-cells, 100%). (C) Individual with Blackfan-Diamond syndrome (Hb F, 4.2%; F-cells, 21.0%).
body. The lower levels of Hb F (10%–20%) in the Greek type of HPFH created several problems in studying the distribution of Hb F among cells with the Kleihauer test; all cells stained for Hb F, but the staining was faint and often difficult to demonstrate without appropriate controls. In contrast, all the cells of heterozygotes for the Greek type of HPFH fluoresced after exposure to fluorescent anti-Hb F antibodies.

(2) Heterozygous β-thalassemia: A wide variety of F-cell levels was observed among heterozygotes for β-thalassemia, approximately half of the values falling above the upper limit of the normal range (Fig. 6). These findings were consistent with those obtained using the acid-elution procedure. Increased numbers of F-cells were present in some, but not all, β-thalassemia heterozygotes.

(3) Sickle cell anemia: The proportions of F-cells in the five black patients with sickle cell anemia (whose Hb F levels fell between 4.2% and 10.1%) ranged from 30% to 50%. In each case a clearly heterogeneous distribution of F-cells was observed. The levels of F-cells in cases of the sickle cell trait are discussed in a separate paper.

(4) Hereditary aplastic anemias: Two families with Blackfan-Diamond syndrome and one individual with Fanconi’s anemia have been studied with this technique. The proportions of F-cells in the patients with the Blackfan-Diamond syndrome increased with increasing amounts of fetal hemoglobin (Hb F ranged from 1.1% to 7.8%) in the peripheral blood (Fig. 6). One affected person did not respond to steroid treatment and received frequent transfusions;
this patient had a normal F-cell level of 3.0%. In the individual with Fanconi’s anemia (confirmed by chromosome studies), the fetal hemoglobin (which constituted 13.5% of the total Hb) was distributed among 55% of his red cells, as determined after dilution of his blood with that of a normal individual.

(5) Acquired conditions: All cases of polycythemia vera thus far examined have exhibited normal F-cell proportions. Increased proportions of F-cells were commonly observed in the acute forms of lymphocytic and myeloid leukemia, but not in the chronic leukemias. No attempt has been made to relate the data presented in Fig. 6 to type of treatment, severity of the disorder, or whether the condition is in remission or relapse; such an attempt would necessitate a separate, carefully controlled study. The levels of F-cells in preleukemic states should also be examined, as the sensitivity of the fluorescent antibody test to small changes in amounts of Hb F could be of diagnostic value.

DISCUSSION

The data presented in this paper indicate that immunofluorescent F-cell labeling is far more sensitive than alkali denaturation in detecting small elevations of Hb F. At low levels of Hb F, the immunofluorescent procedure is also much more sensitive than the acid-elution test of Kleihauer et al.1 With the acid-elution method, it is difficult to detect any stained cells in smears of normal bloods, and evaluation of the results is tedious, requiring carefully matched normal adult and cord blood controls for each estimation. The technique of immunofluorescent identification of F-cells in fixed smears also appears more sensitive than procedures based on immunodiffusion. Greater proportions of F-cells are detected by the application of fluorescent anti-Hb F antibodies to smears from normal adults than are demonstrable by the immunodiffusion procedure of Boyer et al.2 Using a monospecific anti-A2 antibody, the average of 0.75 pg of Hb A2 per cell can be detected in every cell of the normal adult by the application of fluorescent anti-A2 antibody to blood smears; the same antibody cannot detect Hb A2 in single cells using the cellular immunodiffusion procedure (unpublished observations). On the other hand, the immunofluorescent procedure described herein is less satisfactory when the proportion of F-cells exceeds 30%, since, in these cases, there is considerable variation in the extent to which individual cells fluoresce, and it is often difficult to differentiate weakly labeled from unlabeled cells. To obtain accurate F-cell counts in such cases it is necessary to dilute the “high F-cell” sample with blood from a normal individual in whom the F-cell proportion is known. The red cell counts in each sample must also be taken into consideration in the final estimation of the proportion of F-cells in the abnormal sample. The three techniques for identification of Hb F in red cells, i.e., the acid-elution procedure of Kleihauer et al.,1 cellular immunodiffusion,21 and the application of fluorescent antibodies to fixed blood smears, appear to differ in sensitivity and, thus, may have different applications. The high threshold of the acid-elution method makes it superior in the diagnosis of fetal-maternal transfusion. The cellular immunodiffusion procedure provides an opportunity for quantifying the amount of Hb F per cell.21 The simplicity and high sensitivity of fluorescent identification of Hb F in fixed smears may prove useful to the study of hereditary and acquired conditions in which there are small elevations of fetal hemoglobin.
An average of 2.7% (range, 0.5%-7.0%) of the cells of normal adults were found to contain Hb F. This “clonal” appearance of F-cells in the adult is consistent with either of two possible explanations. F-cells might arise from clones of erythroid cells capable of Hb F production. Under circumstances resulting in increased synthesis of Hb F, these clones might be preferentially stimulated to increase their contribution to the erythrocyte population. Alternatively, the amount of Hb F in adult erythrocytes might be normally distributed, and cells containing less than a threshold amount of Hb F might be undetectable by the immunofluorescent technique. In this situation, an increase in the amount of Hb F would produce an upward shift of the mean of the distribution and, hence, an increase in the number of F-cells detected. The immunofluorescent procedure does not provide a basis for quantitation of amounts of Hb F per cell. There is uncertainty about the nature of the relationship between the brightness of fluorescence and the amount of Hb F in a given cell, and measurement of the intensity of emitted light is not feasible, owing to the rapidity with which the FITC conjugates fade. However, if the “clonal” appearance of F-cells in normal adults were simply a threshold effect, one would expect the amounts of Hb F in the detectable F-cells to follow a one-tailed distribution, rather than the normal distribution observed by Boyer et al. upon application of a quantitative immunologic technique.

The significance of the F-cell proportions in excess of 10% in some otherwise normal individuals is, at present, unknown. Complete studies of the families of the two blacks and one white whose F-cell levels fell between 10% and 15% were not possible. However, the mother of one of these black subjects had 8.4% F-cells, while the father’s F-cell proportion was within normal limits (4.2%). It is possible that these otherwise normal persons with F-cells in the 10%-15% range are carriers of the postulated Swiss variant of hereditary persistence of fetal hemoglobin. This anomaly, originally described by Marti and subsequently observed by others, is thought to produce mildly elevated F-cell counts (upon application of the acid-elution test, about 1% of the erythrocytes stain for Hb F) and levels of Hb F ranging from 1% to 3% of the total hemoglobin. No systematic studies of populations or families with the abnormality have been published. The scarcity of family data prevents one from considering the Swiss type of HPFH as a simple Mendelian trait. Until this question is resolved, the reasons for the small increases in F-cell proportions in some hematologically normal adults will remain unclear.

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