Fetal Hemoglobin-containing Erythrocytes I. Counts of Cells Stained by the Acid Elution Method Compared with Alkali Denaturation Measurements

By Kosmas A. KiossoGlu, Irving J. Wolman and Mortimer Garrison, Jr.

There is considerable interest currently with respect to the acid elution method of Kleihauer, Braun and Betke\(^1\) for detecting cytologically those circulating erythrocytes which have an appreciable content of fetal hemoglobin ("F-containing cells"). The method is based on the lower solubility of alkali-resistant or fetal hemoglobin in an acid environment as compared with that of normal adult hemoglobin.

This communication compares the counts of F-containing cells obtained by the acid elution method with simultaneous measurements of alkali-resistant hemoglobin in a variety of hematologic conditions including thalassemia major and minor, aplastic anemia, leukemia and normal controls.

**Methods**

The chemical measurement of fetal hemoglobin was done by the alkali denaturation method of Singer, Chernoff and Singer.\(^2\) All determinations were done in duplicate, and the figures given represent the mean values. The level of circulating hemoglobin was measured photoelectrically by a standard alkali hematin procedure.

The acid elution preparations were made in conformity with the procedure described by Kleihauer, Braun and Betke\(^1\) and Betke and Kleihauer.\(^3\) Smears of fresh blood were collected on clean slides from a finger prick, care being taken to obtain thin films in which the erythrocytes were distributed evenly without overlapping. The slides were air-dried for about 1 hour, fixed in 80 per cent ethanol for 5 minutes, rinsed with tap water, placed vertically in buffered citric acid-phosphate solution (McIlvaine\(^4\)) at pH 3.2 to 3.4, and incubated for 5 minutes at 37 C. The buffer is made from the following stock solutions:

\[ a. \ 0.1M \ \text{solution of citric acid (19.21 \ Gm. in 1000 ml.)} \]
\[ b. \ 0.2M \ \text{solution of dibasic sodium phosphate (53.65 Gm. of Na}_2\text{HPO}_4\cdot7\text{H}_2\text{O or 71.7 Gm. of Na}_2\text{HPO}_4\cdot12\text{H}_2\text{O in 1000 ml.)} \]

Composition: X ml. of a solution plus Y ml. of b solution to a total 100 ml.

<table>
<thead>
<tr>
<th>(a)</th>
<th>(b)</th>
<th>(pH)</th>
</tr>
</thead>
<tbody>
<tr>
<td>75.4 ml.</td>
<td>24.6 ml.</td>
<td>3.2</td>
</tr>
<tr>
<td>71.8 ml.</td>
<td>28.2 ml.</td>
<td>3.4</td>
</tr>
</tbody>
</table>

After incubation the slides were rinsed with tap water, stained with 0.5 per cent aqueous solution of eosin for 3 minutes, rinsed again with tap water, and dried. For best results it is essential to keep the pH precisely at 3.2 to 3.4 and to stain the slides within a few hours after the finger prick. Smears from normal blood, if processed after standing overnight, may give falsely high readings for fetal cells, at times up to 100 per cent.

Red cells which have lost their hemoglobin appear as shattered pale-staining ghost forms, whereas those which contain fetal hemoglobin ("F-containing cells") are largely...
intact and take the eosin stain vividly. There are usually also a few cell forms which do not seem fully disintegrated and stain feebly and irregularly with the eosin. Since traces of retained stain can be prominent visually, and since such forms have represented up to 5 per cent of all cells in some preparations from control subjects whose blood contained no more fetal hemoglobin on chemical measurement than other normal individuals, it was deemed advisable to group these with the non-hemoglobin-containing cells.

Each slide received a preliminary inspection under low magnification (16 mm. objective and x10 eyepiece). Those in which the red cells were not distributed evenly or stained properly were discarded. Under oil immersion 500 red cells were then counted, and the number of F-containing cells reported as a percentage. All counts were done blindly, on coded slides, without the observer being aware of the clinical status of the test subjects.

Lovelock has shown that repeated washings will remove up to 30 per cent of the stromal lipid from oxalated red cells, but only about 2.5 per cent of the contained hemoglobin. Accordingly, 10 samples of oxalated red cells—ranging in their positive staining properties from 3 to 85 per cent—were washed serially in normal saline solution up to 25 times, with 5 minutes centrifuging at 1,000 rpm between each washing. The count of F-containing cells was not altered significantly by these washings, indicating that a positive acid elution response is a reaction with fetal hemoglobin pigment rather than with the lipid structure of the erythrocytes.

The reliability of the acid elution staining and counting was approached in two ways: a) A Pearson product-moment correlation coefficient (r) was computed for the number of F-containing cells reported by two observers who independently had counted the same 75 slides. The correlation between the two sets of observations was .85. b) The same 75 slides were counted again by one observer who in the second study was in ignorance of his previous results. The correlation between these two counts (original and recount) was .88, and rises to .95 when one case is excluded for whom the first count was later found to be in error. With both approaches, the correlations were sufficiently high to demonstrate the reliability and reproducibility of the procedure.

**Experimental Subjects**

A total of 82 individuals were studied: 19 normals and 63 with some sort of blood dyscrasia. There were 17 children and adults with asymptomatic thalassemia minor; 10 children with aplastic anemia of different kinds and degrees of severity; 10 children with lymphocytic and 1 with granulocytic leukemia in assorted phases of remission and relapse; 13 children and 1 adult with thalassemia major (all had been transfused 3 to 5 weeks previously); and 11 with miscellaneous other disorders (1 Wilms' tumor; 1 osteogenic sarcoma; 1 Hodgkin's disease; 1 iron deficiency; 2 acute hemolytic disease; 2 sickle-cell disease; 3 sickle-cell trait).

**Results**

1. **Comparison between methods.** The product moment correlation in 94 pairs of measures of the F-containing cell count and the alkali-denaturation readings from 82 subjects was .79. This correlation is statistically significant; it indicates that a positive relationship exists between the measures but that the degree of agreement, while substantial, is far from perfect. The extent of the agreement is indicated also by the quantitative findings in the diagnostic groups as described below and graphically in figure 1. There is general agreement between methods in the ordering of the group averages from high to low, but considerable quantitative difference occurs in the actual values obtained.
2. Comparison between groups. The values for age and blood hemoglobin levels as well as for F-containing cell counts and alkali resistance measurements reveal significant differences between the groups (table 1). The F-containing cells had their highest mean incidence in thalassemia major (15.5 per cent) and were encountered with diminishing frequency in leukemia (6.27 per cent), aplastic anemia (5.66 per cent), miscellaneous conditions (3.14 per cent), thalassemia minor (2.24 per cent) and controls (0.97 per cent). As a rule, the mean values for alkali-resistant hemoglobin were lower than with the cytologic approach.

3. Ratios. The ratios of the reading for acid-elution over that for alkali-denaturation were highest in leukemia (2.76) and in thalassemia major (2.58); less in aplastic anemia (1.58) and in thalassemia minor (1.42); and lowest in the miscellaneous group (1.02) and in the normals (0.83). In general, the ratio was highest when the fetal hemoglobin content of the red cells was highest as determined by both methods.

4. Relation to age and total blood hemoglobin levels. The thalassemia major, leukemia and aplastic anemia groups were made up essentially of children with reasonably similar age distributions, whereas the thalassemia minor and normal groups contained many adults. To determine whether the F-containing cell count and the alkali-denaturation assay might be affected by the factors of age or total hemoglobin level, these variables were intercorrelated for the whole range of subjects—normal, anemic and miscellaneous, with
Table 1.—Grouped Data from the Test Subjects, Comparing the Fetal Hemoglobin as Measured by Acid Elution (Fetal Cell Count) and by Chemical Analysis (Alkali Denaturation)

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of Subjects</th>
<th>Age (years)</th>
<th>Hemoglobin (Gm. 100 ml.)</th>
<th>Fetal Cell Count (%)</th>
<th>Alkali Denaturation (%)</th>
<th>Acid Elution</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>Range</td>
<td>S.D.</td>
<td>Mean</td>
<td>Range</td>
</tr>
<tr>
<td>Normal</td>
<td>19</td>
<td>21.33</td>
<td>2-48</td>
<td>12.89</td>
<td>14.63</td>
<td>11.3-16.5</td>
</tr>
<tr>
<td>Thalassemia minor</td>
<td>17</td>
<td>29.29</td>
<td>1-55</td>
<td>15.53</td>
<td>12.59</td>
<td>10.8-15.9</td>
</tr>
<tr>
<td>Aplastic anemia</td>
<td>10</td>
<td>9.50</td>
<td>3-14</td>
<td>2.83</td>
<td>11.76</td>
<td>8.3-16.0</td>
</tr>
<tr>
<td>Leukemia</td>
<td>11</td>
<td>7.36</td>
<td>3-13</td>
<td>2.27</td>
<td>11.30</td>
<td>4.2-15.2</td>
</tr>
<tr>
<td>Thalassemia major</td>
<td>14</td>
<td>9.57</td>
<td>1-33</td>
<td>7.91</td>
<td>10.01</td>
<td>8.4-12.7</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>11</td>
<td>11.45</td>
<td>1-38</td>
<td>12.21</td>
<td>10.15</td>
<td>6.1-13.6</td>
</tr>
</tbody>
</table>

*Wilms tumor 1; osteogenic sarcoma 1; Hodgkin's disease 1; iron deficiency 1; acute hemolytic disease 2; sickle-cell disease 2; sickle-cell trait 3.
Table 2.—Intercorrelations Among the Variables

<table>
<thead>
<tr>
<th>Age (years)</th>
<th>Blood Hb Level (Gm.)</th>
<th>% Fetal Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blood Hb Level (Gm.)</td>
<td>+.39</td>
<td></td>
</tr>
<tr>
<td>% Fetal cells</td>
<td>−.30</td>
<td>−.39</td>
</tr>
<tr>
<td>% Fetal Hb by alkali method</td>
<td>−.32</td>
<td>−.41</td>
</tr>
</tbody>
</table>

With Ns of 81 for correlations involving age and 82 for all others, these correlations are all statistically reliable at or better than p = .01.

The means and the wide ranges of readings for fetal hemoglobin in the disorders here reported are in conformity with what have been encountered by other workers. In most other reports, the data have been obtained with either the chemical or the cytologic method. In Babini’s6 studies of 10 premature and full-term infants however, the F-containing cell counts were higher than the results of chemical assay of fetal hemoglobin to approximately the same degree as in our own observations.

Despite the quantitative differences in our results with the two methods, there is a considerable similarity in the findings as indicated by the correlation of +.79 between them. In addition, the correlations between both sets of results and the factors of age and total hemoglobin are quite similar. The trend of the comparative ratios indicates that proportionately more red cells will be counted as F-containing when the total percentage of fetal hemoglobin is high.

One must bear in mind that the two methods of appraising fetal hemoglobin do not measure exactly the same phenomenon. The alkali-denaturation procedure determines with reasonable chemical accuracy the percentage of fetal hemoglobin in the total mass of circulating hemoglobin. The acid-elution procedure rates each cell on an all-or-none basis, and tabulates those which contain sufficient fetal hemoglobin to give a positive staining reaction as judged by specified criteria. If it is true that individual red cells may contain both adult and fetal hemoglobin,7,9 then some sort of a threshold for staining quality may exist, and those cells which respond positively in the acid-elution technic, may have a relatively higher content of fetal hemoglobin. This could help to explain the persistently higher readings found between the two methods in the abnormal conditions surveyed. In some of the diseases studied which are accompanied by anemia (and notably in thalassemia major), anisocytosis, poikilocytosis, microcytosis and hypochromia are present to contribute to the discrepancies, in that the corpuscular content of hemoglobin
in such distorted cells tends to be subnormal. For the different groups here reported the fetal cell count gave lower standard deviations and smoother curves than did the chemical assays in the statistical handling of the data.

The clinical applications of the acid-elution method for the detection of red cells containing fetal hemoglobin will be discussed in a subsequent report. Hence, applications of the data in the different diseases to phenomena exhibited by patients are not discussed here. A number of reports have already appeared on observations with the acid elution procedure in pregnancy,\textsuperscript{10,12} in the newborn,\textsuperscript{12,14} in infancy periods,\textsuperscript{x} and in thalassemias.\textsuperscript{12,13,15,16}

The acid elution procedure requires much less time and equipment, avoids venipuncture (which is important in children), and in many ways lends itself more readily to the facilities of a routine hematology laboratory. Furthermore, as discussed below, it seems more sensitive than the alkali-denaturation procedure that was employed when the concentration of fetal hemoglobin is borderline or slightly elevated.

In the thalassemia minor group, only 2 of the 17 carrier individuals exhibited readings above the 2 per cent level with alkali-denaturation (2 per cent is the accepted top level for normal). In contrast, 12 of the 17 had F-containing cell counts above 1.5 per cent, which was the highest count exhibited by any of the 19 normal controls in our series. These slight but significant elevations are parallel to those observed by Ballerini et al.,\textsuperscript{16} who noted that 47 out of 50 individuals with thalassemia minor had F-containing counts over 1 per cent, with most of the readings between 2 and 5 per cent. Kleihauer and Betke\textsuperscript{12} noted also that individuals with thalassemia minor often show small numbers of F-containing cells in their circulation despite a normal fetal hemoglobin content of the blood with the chemical studies. Thus, for detection of this genetic carrier trait, the F-containing cell count would appear to be more helpful than the alkali-denaturation approach.

Conclusions

The quantitative study of peripheral smears for red cells containing fetal hemoglobin ("F-containing cells") by the acid elution method is a useful and dependable screening test. Findings with this approach were compared with fetal hemoglobin assay by alkali-denaturation in a series of 19 normal subjects and 63 subjects with an assortment of blood disturbances. The results of the two methods had a correlation of +.79, suggesting a reliable but far from perfect identity between the methods. The acid elution method tended to give higher readings, and seemed more sensitive for recognizing individuals with slight increases in red cell fetal hemoglobin as occurs in thalassemia minor.

**Summario in Interlingua**

Le studio quantitative de frottis de sanguine peripheric pro erythrocytos continente hemoglobina fetal per medio del methodo a elution acide representa un utile e fidel test de cribrage. Le resultatos obtenite per iste metodo esseva comparate con le constatationes de studios a denaturation
IIB-F-CONTAINING ERYTHROCYTES 1

559

alcalin in un serie de 19 subjectos normal e 63 subjectos con un varietate de disturbationes hematologic. Le valores determinate secundo le duo methodos monstrava un correlation de +0,79, lo que indica un satisfacente sed certo non perfecte identitate inter le duo methodos. Le methodo a elution acide tendeva a producer plus alte valores e pareva plus sensibile in le recognition de individuos con leve augmentos de fetal hemoglobina erythrocytic, como illos occurre in thalassemia minor.

ACKNOWLEDGMENT

We are indebted to Mr. Kenneth Myers for skillful technical assistance.

REFERENCES

11. Borst-Eilers, E.: The foetal origin of red cells staining with Kleihauer's technique, as established by the application of the "mixed agglutination" reaction on those cells. Vox Sang. 6:451, 1961.
Kosmas A. Kiossoglou, M.D., formerly Instructor in Pediatrics, Graduate School of Medicine, University of Pennsylvania, Philadelphia, Pa.; at present Research Fellow, Blood Research Laboratory, Pratt Clinic—New England Center Hospital, Boston, Mass.


Mortimer Garrison, Jr., Ph.D., Director of Research, Woods Schools, Langhorne, Pa.
Fetal Hemoglobin-containing Erythrocytes I. Counts of Cells Stained by the Acid Elution Method Compared with Alkali Denaturation Measurements

KOSMAS A. KIOSSOGLOU, IRVING J. WOLMAN and MORTIMER GARRISON, JR.