Fetal Characteristics of Erythrocytes in Sickle Cell Anemia: An Immunofluorescence Study of Individual Cells

By Alice Maniatis, Thalia Papayannopoulou, and John F. Bertles

In a group of disease states that includes sickle cell anemia (SS disease), two fetal erythrocyte markers, Hb F and i antigen, persist into adulthood. Using the technique of single-cell immunofluorescence, we determined the expression of I-i antigens and the presence of Hb F within populations of erythrocytes. Subjects tested included normal adults, normal newborns, patients with SS disease, and individuals with sickle cell trait. We classified erythrocytes reacting to anti-i as i cells and those reacting to anti-I as I cells, a terminology analogous to that used to identify erythrocytes containing increased amounts of Hb F as F cells. The expression of I and i antigens within populations of both normal and SS erythrocytes was found to be heterogeneous. The proportions of both i cells and I cells in all SS patients studied exceeded those found in normal adults, and an overall stronger-than-normal reactivity of individual SS cells to the two antibodies was observed. Proportions of F cells showed no correlation with proportions of i cells; and with double fluorescence staining for both Hb F and i, a significant proportion of each total SS red cell population was found to carry only one or the other marker. These findings confirm and clarify on a cellular level our previous demonstration, by means of quantitative hemagglutination, that there is increased expression of both I and i by whole populations of SS erythrocytes. In addition, we provide here new information on the expression of I and i within populations of normal human erythrocytes.

THE I-i ANTIGEN SYSTEM of human erythrocyte membrane has two unusual characteristics: it is involved in the majority of instances of cold agglutinin disease, and it is unique in undergoing a reciprocity in expression early in postnatal life. That is, whereas i is expressed strongly and I weakly on cord red blood cells, a gradual reversal of this antigenic expression occurs that is complete before the age of 2 yr. This gradual diminution in i expression during ontogeny is analogous to the decrease in red cell content of fetal hemoglobin (Hb F) in the first postnatal year.

However, in certain disease states in adults, either i reactivity or the proportion of Hb F is found to be significantly increased, and sometimes both are increased. In studying one of these disease states, sickle cell anemia (SS disease), we found increased expression of both i and I on erythrocytes of adult patients, whereas the expression of ABH antigens was normal. We also documented the absence of any correlation between the level of i expression and the amount of Hb F in the blood of

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these SS patients. The technique used in that study (quantitative hemagglutination) measured average agglutinability by anti-i or anti-I antisera within whole populations of erythrocytes. Hb F was quantitated by the technique of alkali denaturation.

The purposes of this investigation were (1) to determine whether the increased I-i reactivity of SS cells is brought about by increased antigen density on a limited number of cells or by an increased number of cells with near-normal I-i expression and (2) to examine on a cellular level the relationship between Hb F and i by employing a double fluorescence staining technique.

**MATERIALS AND METHODS**

**Blood Samples**

Fresh venous blood, collected in EDTA (2 mg/ml blood) as anticoagulant, was obtained from 30 normal adults, 26 newborns, 15 individuals with SS disease, and 40 individuals with sickle trait (AS).

**Reagents**

The anti-i serum “Becker” (a gift of Mr. W. L. Marsh) has been characterized previously. Anti-i “Den” (a gift of Mrs. M. Crookston) has an agglutination titer of 320 against cord erythrocytes and less than 16 against adult erythrocytes at 4°C. Anti-i “Myerson” (a gift of Dr. P. Lalezari) has a titer of 2000 against cord erythrocytes and less than 40 against adult erythrocytes at 4°C. Anti-Hb F antibodies were prepared, purified to monospecificity, and conjugated with fluorescein isothiocyanate (FITC) or with tetramethylrhodamine isothiocyanate (TRITC). Antiserum to human IgM (μ chain) coupled with FITC or TRITC was purchased from Cappel Laboratories, Downingtown, Pa.

**Immunofluorescence Labeling**

**I-i antigens.** Red cells were washed three times with phosphate-buffered (pH 7.2) saline solution (PBS) (hemagglutination buffer, Difco Laboratories, Detroit, Mich.) and were resuspended in PBS containing 2% human serum albumin (Cutter Laboratories, Berkeley, Calif.). Thin smears on slides were dried for at least 1 hr or overnight, fixed in 100% methanol for 1 min, washed first in PBS and then in distilled H₂O, and air dried. Anti-I or anti-i antiserum at an appropriate dilution was applied to preselected areas on the smear, and the slides were incubated at 4°C for 2 hr in a humidified atmosphere. The slides were then rinsed in cold PBS, followed by distilled H₂O, and were dried. TRITC- or FITC-anti-IgM (diluted 1:5) was applied, and slides were incubated at 16°C for 2 hr, then washed and air dried. Preliminary testing showed these temperature conditions to be optimal. Between 500 and 1000 erythrocytes were scored in each preparation.

**Hb F.** For the detection of Hb F, direct immunofluorescence staining was used. The slides were prepared and stained as previously described. Between 2000 and 3000 cells were scored in each preparation.

**Double fluorescence labeling of Hb F and i.** For this double-labeling procedure, thin-smeared slides were fixed in acetone: ethanol (9:1). This fixative was found to be better for F-cell staining and did not interfere with i-cell staining. Anti-F antiserum conjugated with FITC (or TRITC) was applied to preselected and marked areas of the smears. The slides were incubated for 1 hr at 37°C in moist chambers, washed in PBS followed by distilled H₂O, and dried. Anti-i antiserum was then applied to the same marked areas of the smears, and the slides were incubated at 4°C for 4–5 hr. The washing and drying were repeated, TRITC-anti-IgM (or FITC-anti-IgM) was applied to the same marked areas for 2 hr at 16°C, and the slides were washed and dried. It should be noted that in this double-labeling technique the labels must differ, e.g., if FITC-anti-F is used, then the anti-IgM must be conjugated with TRITC. Reversal of this sequence of labeling, i.e., anti-i followed by anti-F, gave less reproducible results.

**Fluorescence Microscopy**

Fluorescing cells were detected with a Zeiss fluorescence microscope equipped with an epifluorescence system (HBO 50-W lamp) and appropriate filter combinations for FITC and TRITC. Photographs were taken on 35-mm Eastman Tri-X Pan film.
Quantitation of Hemoglobins

The proportions of hemoglobins A, S, A₂, and F in whole-cell populations were quantitated by isoelectric focusing and scanning densitometry. In addition, proportions of Hb F were quantitated by alkali denaturation.

RESULTS

Detection of I Cells and i Cells

Photomicrographs of representative cell preparations are shown in Fig. 1. The intensity of fluorescence varied among cells, but in all preparations one could distinguish cells with strong homogeneous fluorescence, cells with weaker fluores-

Fig. 1. Cord erythrocytes (A and B), adult erythrocytes (C and D), and SS erythrocytes (E and F) stained with anti-i and FITC-anti-IgM (A, C, and E) and with anti-i and FITC-anti-IgM (B, D, and F).
cence and a stippled appearance, and nonfluorescent cells. The contrasting reactions of normal cord cells (A and B) and adult cells (C and D) with anti-i (B and D) or anti-I (A and C) are apparent.

**Anti-I**

A dilution (1:25) of serum "Becker" was chosen that maximized its contrasting reactions with cord and adult erythrocytes. At this dilution the proportions of fluorescing cells in normal adults ranged from 18% to 80% (47.3 ± 20.0% SD) and in normal cord samples from zero to 20% (4.5 ± 4.2% SD). The range in AS samples was 22% to 95% (59.1 ± 20.0% SD) and in SS samples 63% to 90% (78.1 ± 10.6% SD) (Fig. 2). The difference between AA and SS cells was highly significant (p < 0.005).

**Anti-i**

Dilutions of serum "Den" (1:100) and serum "Myerson" (1:50) were chosen to maximize the contrasting reactions of normal cord and adult erythrocytes. Proportions of fluorescing cells when serum "Den" was used are shown in Fig. 3. In normal cord samples, positive cells ranged from 22% to 85% (49.0 ± 17.4% SD); in normal adult samples they ranged from less than 1% to 15.5% (2.2 ± 3.4% SD); in AS samples they ranged from less than 1% to 18% (2.3 ± 3.8% SD); in SS samples they ranged from 4.7% to 51.5% (25.9 ± 14.0% SD). The difference between AA and SS cells was highly significant (p < 0.005). Similar results were obtained with serum "Myerson." Neither anti-i nor anti-I detected a difference between AA and AS erythrocytes. As shown in Table 1, the percentage of i cells in SS patients did not correlate with the reticulocyte count or the degree of anemia.

**Detection of F Cells**

F-cell counts performed on 11 of the 15 SS patients ranged from 6.0% to 45.0% (Table 1). Values for normal adults by this technique have previously been established.
Correlations Between Hb F and i Antigen

As shown in Table 1, although the percentage of F cells correlated well ($r = 0.97$) with the percentage of Hb F, no correlation of percentage F cells with percentage $i$ cells existed.

Double fluorescence staining for Hb F and $i$ (Fig. 4) was performed on the erythrocytes of 4 SS patients. The proportion of red cells carrying a single marker, i.e., Hb F or $i$, in these preparations (Table 2) was similar to that found in preparations with single staining (Table 1), thus indicating that neither of the two labeling procedures interfered with the other's reactions with cells. The data from the doubly stained preparations for these 4 patients (Table 2) provide evidence that although some cells carry both markers, the greater proportion carry either one or the other.

Because of the unusually high proportion of both F cells and Hb F in patient S.W., we examined the blood of S.W.'s mother and father (Table 3). Both parents were AS by the usual criteria, and double heterozygosity for thalassemia and Hb S
Fig. 4. Double fluorescence staining for Hb F and i antigen. Panel A (illuminated with TRITC excitation beam) and panel B (illuminated with FITC excitation beam) are the same microscope field of a slide stained with TRITC-anti-F (A) and then with anti-i followed by FITC-anti-IgM (B). Arrows point to cells that are stained on both A and B. Note that the majority of F cells (panel A) do not appear under these conditions to express i antigen (panel B).
was effectively ruled out by the peripheral blood smear and the relative proportions of Hb A and Hb S. Thus it appears that the father J.A., who had an elevated Hb F level, carried a gene for a condition known as the heterocellular form of hereditary persistence of fetal hemoglobin (heterocellular HPFH). Presumably this gene, along with the gene for Hb S, was transmitted to the daughter, S.W.

**DISCUSSION**

The usual clinical assay for reactions of red cell surface antigens with specific antibodies involves an estimate of relative agglutinability within a cell suspension representing a whole population of cells from one individual. More recently, a method has been introduced that eliminates subjectivity from the assay by measuring directly the proportions of cells agglutinated at various dilutions of specific antiserum. By using this method of quantitative hemagglutination, we have found increased expression of both I and i antigens on red cells from individuals with SS disease.

It is now apparent from results reported here on the reactions of single erythrocytes that agglutinability with anti-I or anti-i demonstrated by whole populations of AA red cells is dependent on a heterogeneous distribution of I and i antigens among the individual cells of any one population of cells. A variation among normal individuals in proportions of cells detected with antibody in our immunofluorescence system is shown by the wide ranges in the percentages of AA adult cells reacting with anti-i (Fig. 2) and AA cord cells reacting with anti-i (Fig. 3).

Limited information is available on the distribution of red cell surface antigens within cell populations of any genotype. Studies with indirect immunofluorescence technically similar to those in this report have revealed a heterogeneity in expression of A antigen on red cells. The heterogeneity of antigen expression was confirmed for the A antigen by immunoelastron microscopy of cells exposed to anti-A and peroxidase-conjugated anti-IgM and for the H antigen by ferritin-labeled anti-H. Heterogeneity of i antigen distribution by fluorescence staining has previously been described in PNH erythrocytes.

**Table 2. Percentages of i, F, and F-i Cells in 4 SS Patients Obtained by Double Fluorescence Staining**

<table>
<thead>
<tr>
<th>SS Patients</th>
<th>F Cells (%)</th>
<th>i Cells (%)</th>
<th>F-i Cells (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P.G.</td>
<td>36.3</td>
<td>33.2</td>
<td>19.0</td>
</tr>
<tr>
<td>C.P.</td>
<td>32.0</td>
<td>11.8</td>
<td>8.0</td>
</tr>
<tr>
<td>M.O.</td>
<td>39.0</td>
<td>29.0</td>
<td>12.7</td>
</tr>
<tr>
<td>W.H.</td>
<td>28.2</td>
<td>45.8</td>
<td>13.1</td>
</tr>
</tbody>
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*Cells reacting with both anti-F and anti-i as percentage of total red cells present.

**Table 3. Hematologic Data and Percentages of F and i Cells in Patient S.W. and Her Parents**

<table>
<thead>
<tr>
<th></th>
<th>i Cells (%)</th>
<th>F Cells* (%)</th>
<th>Hb F (%)</th>
<th>Reticulocytes (%)</th>
<th>Hb S (%)</th>
<th>Hb A (%)</th>
<th>Hb A2 (%)</th>
<th>Hb (g/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient S.W.</td>
<td>36.5</td>
<td>70.7</td>
<td>14.7</td>
<td>10.5</td>
<td>83</td>
<td>2.5</td>
<td>8.8</td>
<td></td>
</tr>
<tr>
<td>Father J.A.</td>
<td>&lt;1</td>
<td>14.4</td>
<td>4.0</td>
<td>1.0</td>
<td>36</td>
<td>57</td>
<td>3.3</td>
<td>15.0</td>
</tr>
<tr>
<td>Mother C.A.</td>
<td>&lt;1</td>
<td>2.2</td>
<td>1.0</td>
<td>0.4</td>
<td>40</td>
<td>57</td>
<td>2.2</td>
<td>12.5</td>
</tr>
</tbody>
</table>

*F-cell counts performed by microscopic immunodiffusion.
The data presented in Figs. 2 and 3, which were obtained from inspection of microscope fields of which a representative section is shown in Fig. 1, show that the increased reactivity of SS cells to anti-I and anti-i demonstrated earlier by quantitative hemagglutination is produced by a greater-than-normal proportion of SS cells that react with anti-I (75% versus 48%) and anti-i (18% versus 2%) antibodies. Whether or not it is the same individual cells that have increased reactivities to both antibodies remains to be determined, for it is not yet technically feasible to perform both of these immunologic procedures on any one cell. In addition, it was noticed that SS samples had consistently higher proportions of bright homogeneously fluorescent cells than AA samples (Fig. 1E and F). There are two possible mechanisms for this increased reactivity to anti-I and anti-i. It could reflect greater density of antibody receptors on the erythrocyte membrane surface. Alternatively, it could be due to the greater availability of antigen molecules without increase in their number. This increased availability might be due to alteration, within the red cell membrane, of the molecules carrying the I-i antigens. However, any membrane damage produced by intravascular wear and tear or repeated sickling does not appear to contribute to the increased I-i reactivity of SS cells. This statement is based on our previous studies, which did not implicate any subpopulation of SS cells separable on the basis of high-speed centrifugation in the increased expression of I-i antigens. Nor in the present study did we observe any correlation between cell morphology and degree of immunofluorescence. SS reticulocytes were no more or less fluorescent than other SS cells, and there was no difference in fluorescence between deformed cells and cells that were still diskoid. We did not find a significantly increased proportion of fluorescing AS erythrocytes, as compared with AA erythrocytes, with either anti-I or anti-i. This stands in contrast to our earlier observations with quantitative hemagglutination. One possible explanation for this inconsistency is an increased density of I-i receptors per AS erythrocyte, a situation that would not alter the proportion of fluorescing cells but would be detectable by quantitative hemagglutination. Single-cell immunofluorescence is qualitative only, and we presently are attempting to obtain quantification of receptor density by electron microscopy of cells reacted with ferritin-labeled antibody.

Hillman and Giblett found an inverse relationship between i reactivity and marrow transit time in certain hematologic conditions, and they postulated the release from the marrow of a red cell population with incomplete conversion of i to I. It is of interest that they did not find a decrease in I antigen expression; in our study, I antigen expression was in fact increased. Increased erythrocyte I-i reactivity is seen not only in SS disease but also in disorders with prolonged marrow transit time, such as megaloblastic and sideroblastic anemias, and in myeloproliferative disorders. Therefore it is unlikely that a simple correlation exists between marrow transit time and I-i antigen expression. The membrane alteration that leads to increased I-i reactivity remains to be defined.

The proportion of SS cells reacting with anti-i (i cells) (Table I) did not correlate statistically with either the proportion of cells reacting with anti-Hb F (F cells) or the proportion of Hb F in whole blood. This would indicate that in SS disease, whatever mechanisms are responsible for increased Hb F production seem to operate independent of those responsible for i reactivity. In addition, the fact that increased i antigenicity and increased Hb F content are not restricted to the same
individual SS erythrocytes demonstrates at the cellular level that red cells in the SS adult may contain increased amounts of Hb F, but in other respects can display adult features. It must be pointed out that SS individuals should not be considered genetically homogeneous when proportions of Hb F are at issue. The Hb F level may be directly influenced by the simultaneous presence of other genes, presumably near the globin-chain loci. Patient S.W. illustrates this point. She received one gene for Hb S from both parents (Table 2), but in addition she received another gene from her father (J.A.) specific for one form of a probable group of abnormalities referred to generically as HPFH of the heterocellular variety. An SS individual similar to our patient S.W. has been described by Stamatoyannopoulos et al. Several factors are operating to produce the unusually high proportion of F cells in these two SS patients. One is the commonly observed tendency for SS erythroid cells to synthesize increased amounts of Hb F. Another is the gene for heterocellular HPFH operating to increase further the net synthesis of Hb F. Finally, a survival advantage is possessed by circulating SS cells carrying increased amounts of Hb F. It should be noted that the proportion of i cells in the blood of patient S.W. was not higher than those in other SS patients.

In our study of SS patients we were able to demonstrate that Hb F and i antigen are not coordinately expressed. However, we do not imply that generalizations can be drawn that are applicable to all situations of increased Hb F synthesis and to the appearance of other fetal markers in adult humans, whether inherited or acquired. Mechanisms may differ, and each case deserves individual study. For example, previously reported studies by Alter et al. in patients with aplastic anemia following marrow transplantation have shown that the regenerating erythrocyte population contains increased amounts of Hb F as well as increased i antigen, as determined by agglutination. Double fluorescence staining in these cases would be of great interest. Such studies in a number of hematologic disorders are in progress.

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