A Simple Laboratory Alternative to Irreversibly Sickled Cell (ISC) Counts

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Irreversibly sickled cells (ISC) are considered to be a hallmark of sickle cell disease, yet their number in peripheral blood smears varies greatly among different homozygous SS patients. This variation has suggested a role for ISC in the varying clinical manifestations of the disease. Efforts to determine the role of ISC have been complicated by the difficulty in standardizing the quantification of these cells. For this reason, we have attempted to develop an alternative method of quantification that would be less variable than the microscopic counting of cells on blood smears. Because ISC are dehydrated dense cells, a measurement based on cell density seemed an attractive alternative approach. Analysis of whole blood samples on a simple, 2-step density gradient, spun in a microhematocrit centrifuge, showed a strong correlation between the proportion of high density cells and the percentage of morphologically identified ISC. Parallel ektacytometric measurements of cell deformability, another parameter that reflects the low water content and high MCHC of ISC, were also strongly correlated with ISC counts. These findings suggest that either of these measurements, sensitive to the special physical properties of ISC, could be used as an objective substitute for the microscopic counting of ISC.

A LONG-STANDING QUESTION in the understanding of the pathophysiology of sickle cell disease has been the role of irreversibly sickled cells (ISC). However, assessment of the clinical implications of these cells has been hampered by the fact that they cannot be reproducibly quantified. The microscopic counting of ISC requires individual, subjective categorization of cell morphology and is thus inherently subject to variation. With this perspective, we have sought to develop an alternative method for quantification of ISC that is based not on morphology, but rather on a property that could be measured reproducibly and objectively. Such a property is the low ion and water content of ISC, which gives the cells a high mean corpuscular hemoglobin concentration (MCHC) and density, as well as low cell deformability.

In the experiments presented here, we studied the relationship between ISC counts, cell deformability and the proportion of high density cells in blood samples from more than 40 patients with sickle cell disease. The high degree of correlation observed among these parameters suggests that either cell density or whole cell deformability could be usefully employed as an alternative to ISC counts.

MATERIALS AND METHODS

Blood was drawn from 35 SS and 5 SC patients, not in crisis, into heparin or EDTA, and measurements were performed within 5–6 hr. For ISC counts, approximately 50 μl of whole blood was exposed to air for 30 min and then fixed in 0.5 ml of 3% glutaraldehyde in 0.05 M phosphate buffer, pH 7.4. Five-hundred cells were counted in a simple 2-step density gradient, spun in a microhematocrit centrifuge, showed a strong correlation between the proportion of high density cells and the percentage of morphologically identified ISC. Parallel ektacytometric measurements of cell deformability, another parameter that reflects the low water content and high MCHC of ISC, were also strongly correlated with ISC counts. These findings suggest that either of these measurements, sensitive to the special physical properties of ISC, could be used as an objective substitute for the microscopic counting of ISC.

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fractions were dissolved in cyanomethemoglobin reagent containing 0.1% Triton X-100 to ensure lysis; the percentage of cells in each fraction was then calculated from the spectrophotometric measurements of hemoglobin in each fraction. This calculation assumes that all cells in a sample had the same hemoglobin content. High resolution, multilayer gradient analysis showed that this was true, except for a small proportion of young cells. Because the mean cell hemoglobin content of these cells was generally more than 90% of that of the mature cells and because the young cells constituted a minor proportion of the total population, the use of hemoglobin to determine the proportion of dense cells was virtually equivalent to the use of cell counts. The dense portion was usually dissolved in 2 ml of reagent, and the upper fraction was dissolved in 4 ml. From these measurements we calculated the proportion of "dense" cells, i.e., those cells that had a density greater than 1.1056 g/ml. The reproducibility of the dense cell determination was high; 12 replicate determinations performed on a sickle cell sample that contained 13.3% ISC gave a value of 28.0 ± 1.3 (SD) for the percentage of dense cells. Preliminary, multilayer gradient analyses showed that a layer of 1.1056 g/ml allowed passage of virtually all ISC, but restrained the vast majority of cells from normal blood samples (Fig. 1, Table I).

Fig. 1. (A) Microgradients used to determine the proportion of high density cells. Duplicate samples from one normal subject (M.N.) and two sisters (F.H. and V.H.) with homozygous SS disease are shown. The dark band between the first Stractan layer (p = 1.1056 g/ml) and the plasma represents the low density fraction, and all the cells below that cell/Stractan interface represent the high density fraction. See Table 1 for the proportions of dense cells, ISC counts, and deformabilities for these samples. (B) High resolution discontinuous Stractan gradients of samples shown in A. The densities of the Stractan layers ranged from 1.070 to 1.115 g/ml, in approximately 0.004 g/ml increments.
RESULTS

We found a very strong correlation between ISC counts and the percent of dense cells for patients who had homozygous sickle cell disease (Fig. 2). This finding is consistent with our previous observation that the vast majority of ISC are concentrated in the high density regions during density gradient centrifugation. It should be noted that the percentage of cells with densities greater than 1.1056 g/ml was higher than the percentage of ISC, suggesting dehydration of some cells that were not morphologically identifiable as ISC. Interestingly, the ISC versus density correlation did not hold for blood samples from patients with SC disease, in which there were substantial numbers of high density cells, but very few ISC.

As expected from our previous finding that MCHC was the major determinant of reduced ISC deformability, a strong correlation between whole cell deformability and ISC counts was also observed for blood samples from subjects who had homozygous SS disease (Fig. 3). As with the density assay, the deformability of SC samples was reduced even in the absence of appreciable numbers of ISC, and this finding is also consistent with cell dehydration unaccompanied by morphological changes.

DISCUSSION

In these studies we have developed and tested a method for quantitating ISC on the basis of their abnormal water content rather than cell morphology. The extraordinary dehydration of these cells increases their buoyant density and decreases their whole cell deformability, both of which can be measured objectively. A strong correlation was found between ISC counts and both the proportion of abnormally dense cells and the cell deformability. This finding suggests that measurements either of deformability or density distribution could be used as an objective alternative to ISC counts. Because of the fact that it uses generally available laboratory equipment, the density assay should be especially useful.

It should be noted that neither density nor deformability measurement directly measure ISC, which are defined morphologically. This fact is exemplified by our measurements of blood samples from subjects who...
had the HbSC disease, which showed substantial elevations in cell density and impairment of cell deformability in the absence of appreciable numbers of ISC. Thus, clinical use of the density assay requires accurate prior diagnosis of the type of hemoglobinopathy present. However, it may be that cell water content and deformability may be more important than cell morphology for certain pathologic processes. Because of the extreme dependence of sickling kinetics and polymer formation on hemoglobin concentration,6 with high MCHC, dense cells might be disproportionately involved in vasoocclusion of the microcirculation, where the deformability of individual red cells is the major determinant of blood flow.7,8 Correlation of the results of the density assay with some kind of clinical severity evaluation should be useful in determining the rheologic implications of cellular dehydration in sickle cell disease. In addition, our findings suggest that comparative studies of SC and SS patients and of their red cells might elucidate the relationship between cell dehydration and abnormal morphology in the homozygous disease.

REFERENCES

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