An Elution Procedure for Visualization of Adult Hemoglobins in Human Blood Smears

By David Kabat

A procedure is described for visualization of normal and mutant adult hemoglobins in human blood smears. After extraction of blood smears with a concentrated potassium phosphate buffer (2.76 M, pH 7.2), erythrocytes that had adult hemoglobins stained bright red with erythrosin, whereas cells that had only fetal hemoglobin appeared as clear ghosts. Analyses of cord blood from newborn infants indicate that, although most erythrocytes contain only Hb F and a few contain only Hb A, many contain both hemoglobins A and F.

SELECTIVE EXTRACTION METHODS have been described for visualizing erythrocytes in human blood smears which contain hemoglobins F1-F2, S1, and M1, and these methods have proven useful for various investigations. I recently began a study of the switch from synthesis of Hb F to Hb A during human development, and I needed a method for selectively staining erythrocytes which contain adult hemoglobins. The technique described here appears successful for this purpose. It is based on the fact that Hb A is less soluble in concentrated salt solutions than is Hb F. After extraction of blood smears with a concentrated potassium phosphate buffer, erythrocytes with adult hemoglobins (A or S) stain bright red with erythrosin, whereas cells which had only Hb F appear as clear ghosts.

MATERIALS AND METHODS

For staining of adult hemoglobins, thin blood smears are obtained in the usual way, either directly from finger puncture or from anticoagulated (heparin, citrate, or Na2EDTA are satisfactory) blood. After drying for 30 min at room temperature, the slides are immersed for 2-3 min at 25° in 2.76 M potassium phosphate buffer, pH 7.2 (24.1 g/100 ml K2HPO4, 18.8 g/100 ml KH2PO4 in distilled H2O). The slides are agitated gently during extraction. Following extraction, the slides are transferred rapidly into 95% ethanol for 10 min, into H2O for 5 sec, and then into methanol for 5 min. They are then rinsed with H2O and are stained for 4 min with 0.1% Erythrosin B (National Aniline Division, Allied Chemical Co.).

Blood cells are stained for Hb F by the method of Kleihauer and Betke.1,2 Semiquantitative analysis of erythrocyte staining intensities was done by the method of Shepard, Weatherall, and Conley.1 A photographic negative was analyzed with a microdensitometer. The microdensitometer readings were in per cent transmittance (the zero point was set on a region lacking any cells and the 100 point was set on an unexposed film region). The lightest 4 sq μ of cell area was used as a measure of the cell's staining intensity.

The proportion of Hb F in hemoglobin solutions was measured by a microanalytical adaptation of the alkali-denaturation method of Jonxis and Huisman.3

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RESULTS AND DISCUSSION

Figures 1 and 2 show typical staining results with erythrocytes from a normal adult and from the umbilical cord blood of a newborn baby, respectively. Whereas cells from the normal adult stain uniformly and intensely, those from the cord blood appear strikingly heterogeneous. Certain cells stain brightly, whereas others fail to stain and appear ghostlike. The membranes of the ghosts are collapsed, and they frequently appear quite shrunken. Erythrocytes from patients with sickle cell anemia also stain intensely and uniformly with this method.

Surprisingly, the cells in cord blood that stain with this technique appear rather uniform in intensity. Although variations in intensities do occur, particularly when different microscopic fields are compared, there is always a dramatic difference in appearance between cells which stain and those which do not. Figure 3 shows some microdensitometric data which support this conclusion. There is a clear difference between stained and unstained cells, and very few cells fall into the intermediate range.
Fig. 3. Semiquantitative distribution of staining intensities of cord blood erythrocytes, examined after the high salt extraction procedure. The abscissa is the per cent transmittance of a photographic negative; the more intensely stained cells have the higher values. The cells clearly fall into two groups.

The results described above suggest that erythrocytes with adult hemoglobins are stained by this method, whereas those with only Hb F appear ghostlike. Apparently, fetal hemoglobin is extracted from cells which lack Hb A. Assuming that this is true, the proportion of cord blood cells that contains Hb A varies from 20%-45% in different samples. On the other hand, the proportion of cord blood cells that contains Hb F (as evidenced by the staining method of Kleihauer and Betke') is generally in the range of 90%-97%. Therefore, as was concluded previously by others,7,10-12 many of the cord blood cells must contain mixtures of hemoglobins F and A. In fact, my results indicate that the majority of cord blood cells which contain Hb A (generally, about 80% of these cells) also contain Hb F.

Blood cells were also examined from an adult heterozygous for hereditary persistence of fetal hemoglobin. Her blood contained 40% Hb F. This Hb F was uniformly distributed among erythrocytes, as is characteristic of this condition.7 All of her cells likewise stained strongly and uniformly by the present method. Accordingly, the presence of Hb F in erythrocytes in amounts up to 40% of the total does not interfere with their staining for adult hemoglobin by the present method.

The conclusion that the cells which stain contain Hb A is consistent with the fact that this hemoglobin is relatively insoluble in concentrated salt solutions.6 Additional evidence was obtained by direct analysis of the hemoglobin which remained in cord blood cells following the extraction procedure. This hemoglobin was eluted into H2O and was analyzed by an alkali-denaturation technique.8 Whereas the whole cord blood contained 11% Hb A, the retained hemoglobin contained 53% Hb A.

Although some of the Hb F which remains in the extracted cells probably occurs entrapped in overlapping cells in the smear, the majority is presumably coprecipitated with Hb A in cells which contain both types of hemoglobins. Intracellular coprecipitation of different hemoglobins would be expected; it is unreasonable to anticipate that Hb F could be selectively solubilized from the interior of dried aggregates which contain both types of hemoglobins. Accordingly, cells from the adult heterozygote for hereditary persistence of fetal hemoglobin contained the same proportion of Hb F before and after the extraction procedure. Therefore, the finding that Hb F is, to some extent, retained in cord blood cells following the high salt extraction is fully consistent with the conclusion that many cord blood cells contain mixtures of hemoglobins A and F.

One of the most striking features of cord blood smears stained by this method is the similarity in intensity of different stained cells in the same field. The visual
difference between the red-stained and the colorless ghost cells is clear and unmistakable. This would not be expected if the genes for adult globins (i.e., for β and for δ chains) were only gradually derepressed in cells of developing erythroid clones. In that case, one would expect a very heterogeneous distribution in amounts of Hb A in cord blood erythrocytes. The quantities of Hb A would be expected to range between 0%-100%, and one would expect no clear borderline between "stained" and "unstained" cells. On the contrary, these classes of cells are very distinctive (Figs. 2 and 3), and the intensities of the stained cells appear to be distributed over a relatively narrow range. This is compatible with the idea that the genes for adult globin chains may be switched on to a discrete or "quantal" extent, rather than gradually and to a variable degree. Additional evidence which supports this conclusion will be reported elsewhere.¹³

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