Measurement of Red Cell Enzymes in Newborn Infants*

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ABSTRACT

Red cell enzyme activity was measured in packed red cells obtained from spun hematocrit tubes and compared with results obtained using standard methods (washed, filtered, red blood cells [WFRBC]) in order to evaluate the feasibility of using heel puncture as a means of obtaining accurate quantitative red cell enzyme data in the newborn period. Glucose-6-phosphate dehydrogenase (G-6-PD) and pyruvate kinase (PK) were studied in 10 adults, and G-6-PD, PK, phosphofructokinase (PFK), and phosphoglycerate kinase (PGK) in 10 cord bloods.

After centrifugation of the hematocrit tubes, the plasma and buffy coat and clay were removed by filing, and red cells for enzyme assay were sampled from either the top (or “plasma-end”) or the bottom (or “clay-end”) of the packed red blood cells remaining in the microhematocrit tube.

The activities of PK and G-6-PD were significantly higher in the upper or “plasma-end” (P) of red cells sampled from spun hematocrit tubes in adults and cord blood. Red cell PFK activity was also higher in P than in the bottom or “clay-end” (C) in cord blood; PGK was similar in P and C. Thus, the site of sampling from hematocrit tubes affects results, not only for the age-dependent enzymes, but in cord blood for certain non-age-dependent enzymes such as PFK, as well.

Red cell PK activity was significantly different in spun hematocrit tubes and WFRBC in adults and cord blood, with the exception of PK activity in P and WFRBC in adults which were not significantly different. However, G-6-PD, PFK and PGK activities were not statistically different in red cells obtained from the “clay-end” of hematocrit tubes and WFRBC. There was no evidence of white cell contamination affecting total red cell enzyme activity. Thus, it appears from these studies that red cell G-6-PD and most glycolytic enzymes can be accurately measured in newborns by sampling from the “clay-end” or bottom portion of the packed red cells in spun hematocrit tubes obtained via heel puncture.

Introduction

Red cell enzyme determinations are often technically difficult in newborn...
infants owing to the inability to obtain sufficient quantities of anticoagulated blood. Cord blood is ideal since a relatively large volume can be obtained with no risk to the infant. Unfortunately, it is rare that a congenital red cell enzyme deficiency is suspected at birth; thus, cord blood is either not available when diagnostic studies are to be performed or is clotted. Alternatively, the laboratory is often sent a small volume of anticoagulated blood. However, after processing to remove white cells and platelets, the amount of packed red cells remaining is not sufficient to assay the enzymes requested. Spinning blood in hematocrit tubes and cutting off the plasma and buffy coat layers provides a means of obtaining greater quantities of packed red cells. This method, however, has the potential risk of producing inaccurate results owing to either spurious elevation of red cell enzyme activity secondary to contamination with white cells and platelets or to the effects of aging on red cell enzyme activity, i.e., removing red cells for assay from the upper (or "plasma-end") or the bottom or ("clay-end") of the hematocrit tube, which could select for a relatively "younger" or "older" red cell population with higher or lower enzyme activity, respectively.

For these reasons, a study was performed comparing red cell enzyme activity obtained from heparinized venous blood that was either: (1) not processed (unwashed, unfiltered) and spun in hematocrit tubes, which was felt to simulate blood obtained via skin puncture; (2) washed, filtered, and spun in hematocrit tubes; or (3) washed, filtered, partially packed red cells (resuspended in buffered saline [BSG]) which represents the standard method of preparation in our laboratory.

Both glucose-6-phosphate dehydrogenase (G-6-PD) and pyruvate kinase (PK) activities were measured from both upper or "plasma-end" (P) and the bottom or "clay-end" (C) of packed red blood cells obtained from spun hematocrit tubes in adults and in cord blood in order to evaluate statistical variation in enzyme activity attributable to "young" cells versus "old" cells, respectively. The possible contribution of contaminating white cells to red cell enzyme activity in spun hematocrit tubes was indirectly evaluated by comparing enzyme activity from the upper or "plasma-end" (P) of the unprocessed blood (which is the end closest to theuffy coat) to the upper or "BS-end" of the washed, filtered blood (which was essentially white cell free). In addition, phosphofructokinase (PFK) and phosphoglycerate kinase (PGK) activities were studied in cord blood since red cell PFK activity is lower and PGK activity is higher in newborn infants and these differences are accentuated in the "older" cells, or those produced earlier in gestation, which may influence results obtained from the "clay end" of the hematocrit tube.

In this manner, the feasibility of measuring red cell enzyme activity using packed red cells obtained from spun hematocrit tubes was evaluated.

Materials and Methods

Venous blood from 10 adult volunteers and 10 cord bloods was collected in tubes containing dried sodium heparin.* The blood was mixed and spun in hematocrit tubes in a Clay-Adams micro-hematocrit centrifuge for five minutes and was referred to as "unprocessed RBC's", since they were not washed or filtered. The remaining blood was centrifuged at 400 rpm in a PR-J (IEC) centrifuge at 4°C. The white cell and platelet-rich plasma were removed. The cells were then diluted in at least five parts cold buffered saline glucose (BSG) (buffered with PO4
to pH 7.4, containing 200 mg per dl of glucose) and filtered three times at 4°C through double layers of Whatman no. 2 filter paper. After each filtration, the resuspended cells were centrifuged with removal of the residual buffy coat, and the process was then repeated. The red cells were then washed twice in BS and either reconstituted in buffered saline (BS) to a hematocrit of 70 to 80 percent (WFRBC) or spun in hematocrit tubes. Cells prepared in this manner were essentially white cell free.

The plasma and buffy-coat layers were removed by filing the hematocrit tube just below the buffy-coat and snapping the glass. The clay was also filed off. Assays were then performed by removing 10 μl of packed red cells from the bottom or “clay-end” and the upper or “plasma-end” (unprocessed blood) or “BS-end” (processed blood). The microhematocrit tubes contained approximately 60 μl of whole blood. Care was taken to leave at least 10 μl of packed red cells in the tube so that additional hematocrit tubes are used in severely anemic subjects with a low packed red cell volume. A hematocrit of 100 percent was used to calculate enzyme activity in packed red cells obtained in this manner. The hematocrit was measured in the washed, filtered, packed red cells that were resuspended in BS (WFRBC).

Unprocessed red cells were assayed the same day that the blood was obtained. Adults were divided into two groups: in six adults, the washed, filtered red cells were assayed within 24 hrs; in four adults, the processed blood was kept at 4°C for 72 hrs before it was evaluated. Red cell enzyme activity in washed, filtered cord red cells were measured within 24 hrs.

Red cell G-6-PD and PK were assayed by the method of Piomelli et al. This procedure has been modified so that hemolyzing solutions rather than water are used to prepare the hemolysates. In the G-6-PD assay, a 1:1000 dilution of the assay buffer is used as the hemolyzing solution; in the PK assay, 5 mM (n-Tris hydroxymethyl) methyl-2-aminoethane sulfonic acid buffer, pH 7.4, containing 1 mM EDTA and 1 mM mercaptoethanol is used. Red cell PFK was assayed by the methods of Chapman et al, with the substitution of a triethanolamine buffer; PGK was assayed as described by Loder and DeGruchy. A freeze-thaw hemolysate was used for the PFK assay. All enzymatic measurements were performed at 25°C at 340 nm in a Gilford model 2000 recording spectrophotometer. Enzyme activities were expressed as μmoles of substrate converted per 100 ml RBC per min at 25°C. Data were analyzed using the paired t-statistic evaluation. Results were expressed as the mean ± standard deviation.

Results

Hemolysates prepared from the upper portion of packed red cells obtained from spun hematocrit tubes are referred to as “P” (from the “plasma-end” of unprocessed blood) or “BS” (from the “buffered-saline [BS]-end” of washed, filtered blood). Hemolysates obtained from the bottom fraction or “clay-end” are referred to as “C”. Red cells that were washed, filtered and resuspended in BS to a hematocrit of 70 to 80 percent are referred to as “WFRBC”.

Adults

Pyruvate Kinase (PK) (Figure 1): Red cell PK activity was significantly higher in P and BS when compared to C in both unprocessed and washed, filtered RBC’s. Red cell PK activity was also significantly different in C in both unprocessed and

* S. Piomelli, personal communication.

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Figure 1. Red blood cell enzyme activity (mean ± S.D.) from either end of spun hematocrit tubes compared with red blood cells prepared in a standard manner (WFRBC).

Washed, filtered RBC's and BS when compared to red cells prepared in the standard manner (WFRBC). There was no statistical difference in PK activity between P and BS or between P (unprocessed blood) and WFRBC. Results obtained when red cells were washed, filtered and assayed within one day of the unprocessed red cells (“A”) were similar to those obtained when red cells were assayed three days after the unprocessed blood (“B”).

Glucose-6-phosphate dehydrogenase (G-6-PD): (Figure 2): There was a statistically significant increase in G-6-PD activity in P and BS when compared to C in “A” and between BS and C in “B”, but there was no significant difference between P and C in “B”.

There was no significant difference in G-6-PD activity between P and BS. There was also no statistically significant difference between WFRBC and C in both unprocessed and washed, filtered RBC’s. Similar results were obtained in A and B.

Cord Blood

Pyruvate Kinase (PK): (Figure 3): Red cell PK activity was significantly higher in P and BS when compared to C. There was also a statistically significant difference in activity when either P, BS or C were compared with WFRBC. There was no significant difference in PK activity between P and BS.

Glucose-6-phosphate Dehydrogenase: (Figure 3): Similar to PK, G-6-PD activity was significantly higher in the upper or “younger” end (P and BS) when compared to either C or WFRBC. There was no statistically significant difference, however, between G-6-PD activity in C
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<table>
<thead>
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<th>Glucose-6-phosphate dehydrogenase (Units/100ml RBC)</th>
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<td>A. RBC's Washed, Filtered and Assayed Within 1 Day (n=6)</td>
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<tr>
<td>Unprocessed RBC's</td>
</tr>
<tr>
<td>Plasma</td>
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<tr>
<td>Clay</td>
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<td>WFRBC:</td>
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B. RBC's Washed, Filtered and Assayed 3 Days Later (n=4)

| Plasma                               | 218.3 ±3.3             | 215.5 ±14.5             |
| Clay                                 | 211.6 ±8.0             | 207.1 ±10.7             |
| WFRBC:                               | 204.1 ±11.3            |                        |

Figure 2. Red blood cell enzyme activity (mean ± S.D.) from either end of spun hematocrit tubes compared with red blood cells prepared in a standard manner (WFRBC).

Discussion

In studying red cell enzyme activity in newborn infants and young children, screening tests are often used owing to the ease of performing these studies and the small amounts of blood required. In many instances, however, especially in subjects with a young red cell population, these results can be misleading and quantitative assay is desired. The present study has investigated the feasibility of performing quantitative red cell enzyme assays from spun hematocrit tubes containing unprocessed (unwashed, unfiltered) blood.

Whenever red cell enzyme activity is evaluated, great emphasis is placed on removing white cells and platelets owing to the contribution of these cells to...
Figure 3. Red blood cell enzyme activity (mean ± S.D.) from either end of spun hematocrit tubes compared with red blood cells prepared in a standard manner (WFRBC).

Total activity. Differences in enzyme activity have been demonstrated by comparing enzyme activity in whole blood with defibrinated, washed, filtered red cells\(^\text{10}\) or by comparing removal of the buffy coat after washing the red cells, with red cells that are platelet and white cell free.\(^\text{2}\) In these studies the most significant contribution of white cells to total enzyme activity was demonstrated in subjects that were enzyme deficient,\(^\text{7,10}\) not normal controls. In our studies, white cell contamination was also of concern, since unprocessed blood was spun in hematocrit tubes that were cut below theuffy coat layer in order to remove the plasma, platelets and white cells as completely as possible. The contribution of white cells and platelets to total red cell enzyme activity was indirectly assessed by comparing enzyme activity obtained in RBC's sampled from the upper, or "plasma-end" of spun hematocrit tubes (unprocessed blood), which is the end closest to the buffy coat, with the upper or "BS-end" (blood that was washed and filtered) which is essentially free of white cells. There was no statistically significant difference in PK activity between the "plasma" and "BS-ends" in adults or cord blood or in G-6-PD activity in adults. Thus, it appears that spinning blood in hematocrit tubes and cutting the tube below thebuffy coat removes sufficient quantities of white cells and platelets to permit ac-
accurate measurement of red cell enzyme activity in normal newborns and adults. Red cell PK and G-6-PD activities were evaluated in normal adults and in cord blood since these two enzymes represent the most frequently studied and are “age-dependent” enzymes, which implies that PK and G-6-PD activities decline progressively as the red cell ages. Thus, the activities of the age-dependent red cell enzymes taken from spun hematocrit tubes may vary depending on whether the sample is taken from the upper or “plasma (or BS)-end” of a hematocrit tube which may produce significantly higher enzyme activity than sampling from bottom or the “clay-end” (“older” red cells). In the present study, both PK and G-6-PD did demonstrate age-related differences in enzyme activities in spun hematocrit tubes, with significantly higher activity in the red cells sampled from the “plasma” and “BS-ends” when compared to the “clay-ends” in adult and cord blood. Activity of PK was also significantly different in spun hematocrit tubes when compared with red cells prepared in the standard manner [washed, filtered and resuspended in BS (WFRBC)] in adult and cord blood. However, there was no significant difference in G-6-PD activity obtained from the “clay-end” of spun hematocrit tubes containing unprocessed blood and WFRBC and adult and cord red cells. Thus, the most age-dependent enzyme, PK which has a \( t_{1/2} \) of approximately 29 days cannot be accurately measured from the “clay-end” of spun hematocrit tubes containing unprocessed red cells in adults and newborns. However, the less age-dependent enzyme,
G-6-PD, which has a $t_{1/2}$ of 62 days\(^{10}\) can be assayed in this manner in adults and newborns. It can probably be inferred that the other age-dependent enzymes, hexokinase ($t_{1/2}$ of 33 days)$^9$ and aldolase ($t_{1/2}$ of 77 days)$^9$ would yield results similar to PK and G-6-PD, respectively.

The non-age-dependent red cell enzymes should have similar activity in the upper ("plasma or BS-end") and bottom ("clay-end") of hematocrit tubes and, in general, the sampling site should offer no problems. In cord blood, however, there are unique differences in the activities of certain non-age-dependent enzymes when compared to both normal adults and subjects with reticulocytosis, such as increased activity of PGK, enolase (ENO), phosphoglucone isomerase (PGI), and glyceraldehyde-3-phosphate dehydrogenase (G-3-PD), and decreased activity of PFK.\(^{4,5,8,11,12}\) These differences were found mainly in the "older" red cell fractions and were attributed to red cells produced earlier in gestation.\(^4\) Thus, it is conceivable that in newborns, the activity of the non-age dependent enzymes PGK, ENO, PGI or G-3-PD could be higher and PFK could be lower in the bottom or "clay-end" when compared to the upper or "plasma or BS-end." Both PFK and PGK were evaluated in the present study. Activity of PFK was significantly increased in the red cells sampled from the "plasma" and "BS-ends" when compared to the "clay-ends" of spun hematocrit tubes, but the slightly increased mean activity of PGK in the "clay-end" was not statistically significant. There was also no statistically significant difference in PFK and PGK activity between the "clay-ends" of unprocessed blood and WFRBC. Thus, it appears that unlike adults, the sampling site from spun hematocrit tubes may affect results obtained for certain non-age-dependent enzymes like PFK, in cord blood. However, enzyme activity obtained from red cells taken from the bottom or "clay-end" of spun hematocrit tubes containing unprocessed cord blood yields results that are similar to those obtained by standard methods. Similar results would probably be obtained for PGI, G-3-PD, and ENO.

It appears from these studies that red cell glycolytic enzymes and G-6-PD can be accurately measured in newborns from blood obtained from the bottom or "clay-end" of spun hematocrit tubes containing unprocessed blood which is similar to blood obtained via skin puncture. Only the most age-dependent glycolytic enzyme, PK (and probably HK) could not be evaluated in this manner. If an estimate of PK activity is desired from a heel stick specimen in an infant, it is suggested that the upper portion or "plasma-end" be used, since there was no statistical difference between PK activity in the "plasma-end" (unprocessed blood) and WFRBC in adults. In addition, PK activity in eight subjects with reticulocytosis was also studied, and there was no statistical difference in PK activity between the "plasma-end" and WFRBC ("plasma-end" unprocessed blood: 447.2 ± 82.8 units per 100 ml RBC; WFRBC: 432.0 ± 107.5; $p > 0.1$).

This study offers a rational alternative to either screening tests or drawing large quantities of blood in the newborn period. Red cell enzyme activity can be quantitatively measured and a preliminary diagnosis of a specific enzyme deficiency can be made using heel stick blood. Results could then be confirmed and/or more elaborate kinetic data obtained at a later time using more standard techniques.

References

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