Formation of Intracellular Vesicles in Neonatal and Adult Erythrocytes: Evidence against the Concept of Neonatal Hyposplenism

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ABSTRACT. Intraerythrocytic vesicles accumulate in the peripheral blood as a result of impaired clearance of these intracellular inclusions by the spleen. The observation that neonates demonstrate an increased percentage of erythrocytes containing these vesicles constitutes the primary evidence supporting the concept that the newborn is functionally hyposplenic. Neonatal erythrocytes also demonstrate an increased propensity to undergo a variety of endocytic processes. We therefore questioned whether the increase in red cell vesicles in the neonate might be the result of increased vesicle formation as opposed to impaired splenic clearance. Newborn and adult erythrocytes were incubated *in vitro* in synthetic medium at 37° C. Several parameters confirmed the maintenance of physiologic conditions, including levels of erythrocyte phosphate metabolites monitored by nuclear magnetic resonance. The acquisition of intraerythrocytic vesicles during the course of these incubations was compared. Over a period of 144 h, 19.2% of neonatal erythrocytes acquired vesicles compared to 3.7% of the adult cells ($p < 0.001$). The increase in vesicles was greater in younger density-separated erythrocytes in both the neonate (37.6%, $p < 0.0005$) and the adult (10.3%, $p < 0.002$), but persisted even in the oldest erythrocytes (12.2% and 2.4%, respectively). We conclude that the increase in erythrocytic vesicles in the neonate may not simply be an indication of hyposplenism, but a reflection of increased vesicle formation which overwhelms the clearance capability of the spleen. (*Pediatr Res* 24:703-708, 1988)

Abbreviations

G6PD, glucose-6-phosphatase dehydrogenase
MCV, mean corpuscular volume
MCHC, mean corpuscular Hb concentration

The adequacy of splenic function in the neonate is important because of the unique role of the spleen in preventing bacterial septicemia (1-3). Splenic function in the neonate has been considered impaired based upon the finding of several intraerythrocytic inclusions, including Howell-Jolly bodies (3-5), Heinz bodies (6, 7), and intracellular vesicles (7-9) in the peripheral blood of newborns. The spleen normally clears these inclusions from circulating erythrocytes, and failure to do so has been interpreted as a sign of impaired splenic function. The increase in intraerythrocytic vesicles is considered the most specific of

these indicators of hyposplenism. It constitutes the critical evidence upon which the concept of neonatal hyposplenism was first recognized and upon which it continues to be based.

Neonatal erythrocytes exhibit an increased propensity to undergo a variety of endocytic phenomena which are unrelated to the function of the spleen (10-15). We questioned whether the increase in erythrocytic vesicles in neonates was due to enhanced vesicle formation as a consequence of endocytosis and not to diminished splenic clearance. We developed a model which demonstrates that adult erythrocytes acquire intracellular vesicles spontaneously during incubation *in vitro* (16). This model was used to compare the ability of neonatal and adult erythrocytes to undergo spontaneous vesicle formation to determine if the increase in intraerythrocytic vesicles in the neonate could be due to enhanced vesicle formation.

MATERIALS AND METHODS

Heparinized venous blood was obtained from normal adult human donors and umbilical venous blood from normal full-term neonates after obtaining informed consent according to a protocol approved by the Children's Hospital of Buffalo Institutional Review Board. White cell, red cell, and platelet counts were performed using a Coulter S⁺IV analyzer (Coulter Electronic, Hialeah, FL). Reticulocyte counts (17) and G6PD assays (18) were performed using standard techniques. Samples for *in vitro* incubation were obtained from 10 neonates and 10 paired adult controls. Additional samples were obtained from 10 other normal newborns, 10 surgically asplenic children, and 20 children with functional hyposplenism due to a variety of disorders (including sickle cell disease, inflammatory bowel disease, the "born-again" spleen syndrome, and postsplenic irradiation) (2). These 20 children with functional hyposplenism were selected from a group of 66 to match as closely as possible the percentages of vesiculated erythrocytes found in the 20 normal newborns.

Separation technique. After removing an aliquot of unseparated blood for incubation, the remainder of the sample was separated using a density gradient consisting of a 60% aqueous solution of meglamine diatrizoate (Renografin 60, E.R. Squibb & Sons, Princeton, NJ) and an aqueous suspension of colloidal silica particles coated with polyvinylpyrrolidone (Percoll, Sigma Chemical Co., St. Louis, MO) according to the a modification of the method of Vettore *et al.* (19). The gradient was prepared using 35 ml Percoll, 20 ml Renografin 60, and 45 ml distilled water which has a specific gravity of 1.111. One-half milliliter of packed erythrocytes was resuspended in 10 ml gradient and then centrifuged at 35,000g in a 4° C angle-rotor centrifuge. After centrifugation, the cells separated into numerous small layers. The uppermost two-three layers, several of the layers in the middle of the gradient, and the lowermost two-three layers were separated and each washed four times in cold saline.

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Incubation technique. The samples derived from the gradients as well as the original unseparated aliquot of blood were washed in incubation medium leaving a final concentration of white cells less than 0.4×10^9 /liter. This incubation medium consisted of Eagle's Minimum Essential Medium with Earle's salts but without sodium bicarbonate and L-glutamine (GIBCO Laboratories, Grand Island, NY). The medium also included bovine serum albumin (50 mg/ml) and HEPES buffer (428 mg/ml), penicillin (1000 U/ml), streptomycin (50 mg/ml), and the pH was adjusted to 7.4. The washed erythrocytes were resuspended in a final concentration of 1% in 30 ml of incubation medium and were incubated at 37° C in closed polypropylene containers without agitation (16). Every 48 h, the supernatant incubation medium was replaced with fresh medium and a sample was removed for quantitation of vesicle formation. The incubation was continued for 144 h. Each sample was incubated in duplicate, and the final result was expressed as the average of the two duplicates.

Monitoring of incubating erythrocytes. Several parameters were monitored to confirm that incubating erythrocytes were maintained in as close to a physiologic state as possible throughout the 144-h incubation (16). Nuclear magnetic resonance was used to monitor the metabolic status of the erythrocytes over time in a nonperturbing fashion (20). Spectra sensitive to ^{31}P were used to measure the concentration of all soluble phosphorus-containing metabolites. Erythrocyte crenation was evaluated by determining the percentage of 500 red cells in a wet preparation which demonstrated any degree of crenation using an interference contrast microscope (Carl Zeiss, Oberkochen, W. Germany) at 1000 $\times$ magnification (21). Hemolysis during the incubation was measured by comparing the amount of hemoglobin present in the supernatant medium with the amount present in the total sample at the beginning and end of the incubation (22).

Quantitating vesicle formation. Vesicle formation was measured using interference contrast microscopy (16, 21). Apparent erythrocyte surface pits visualized with this method are actually intracellular vesicles (23–25). Five hundred consecutive fixed red

cells were examined in a wet preparation, and the percentage of erythrocytes containing any intracellular vesicles (the "vesicle count") was derived (Fig. 1) (16). This quantitation was performed on all incubated samples every 48 h and on all other samples examined for Howell-Jolly bodies. Incubated erythrocytes were also examined using transmission electron microscopy as described previously (16).

Quantitating Howell-Jolly bodies. Quantitation of Howell-Jolly bodies was performed on all unseparated samples before incubation, on 10 additional normal full-term neonates, on 10 children who were surgically asplenic, and on 20 patients with functional asplenia who were matched as closely as possible for percentage of erythrocytic vesicles with the 30 normal neonates. Howell-Jolly bodies were quantitated using Wright-stained peripheral blood smears examined under light microscopy to determine the percentage of 10,000 erythrocytes containing these inclusions.

Statistical analysis. For the purpose of statistical analysis, the acquisition of vesicles during each 48-h interval of incubation (as well as the entire 144-h period) was calculated using the following formula: $(\text{vesicle count}_{t_1} - \text{vesicle count}_{t_0}) / (100 - \text{vesicle count}_{t_0})$, in which t_1 = vesicle count at the end of the 48-h interval and t_0 = vesicle count at the beginning. Expressing the data in this form allowed each time interval to be statistically independent by eliminating cells from the analysis which already contained vesicles and therefore could not be counted as acquiring additional vesicles in later time intervals. Differences in the acquisition of pits over time in a group of patients was analyzed using the 1-way analysis of variance. The Fisher's least significance test was used to compare specific differences among individual groups. Comparisons between the adult and neonatal samples over all the points in time were analyzed using the 2-way analysis of variance. The student's t test was used to compare differences among isolated paired groups. The relationship between the vesicle count and Howell-Jolly bodies in the various clinical groups was assessed using correlation coefficients. Statistical significance was assumed if the p value was < 0.05 .

Fig. 1. Neonatal erythrocytes after 144 h of incubation *in vitro* examined under interference contrast microscopy at 1000 $\times$. Several of the red cells contain apparent surface "pits," which actually represent intraerythrocytic vesicles. The larger arrow identifies a fairly large vesicle; the smaller arrow demonstrates a relatively small one. The appearance of these vesicles is identical to those found in incubated erythrocytes of adults.

RESULTS

Before incubation, reticulocyte counts, erythrocyte indices and G6PD levels were measured in both unseparated as well as density-gradient separated samples from both neonates and adults (see Table 1). The reticulocyte counts and the percentage of erythrocytes containing vesicles of the unseparated neonatal cells were significantly greater than those of the adult samples before incubation ($p < 0.01$ and $p < 0.001$, respectively). The results of the *in vitro* incubations of unseparated erythrocyte samples are detailed in Table 2. Separate analysis of the adult and neonatal samples demonstrated significant increases in the percentage of erythrocytes acquiring vesicles throughout the course of the 144-h incubations in both ($p = 0.015$ and 0.004 , respectively). When the acquisition of vesicles of the adult and neonatal cells were compared, the neonatal cells acquired greater numbers of vesicles throughout the incubation period ($p < 0.001$).

Adult and neonatal red cells were separated over density gradients. Samples from the least and most dense fractions, as well as from the middle fractions, were examined. Neonatal cells were distinguished by their higher range of reticulocyte counts, MCV, and G6PD activity in each of these fractions as well as in the original unseparated sample (see Table 1). The reticulocyte counts were greatest in the higher (least dense) fractions for both the neonatal ($p < 0.0005$) and the adult ($p < 0.002$) separations. The reticulocyte counts of the three fractions of neonatal cells were significantly greater than those in the three separated fractions of adult cells ($p < 0.005$). Comparative values for the MCV and G6PD assays were also higher in the neonatal samples, whereas the MCHCs were not significantly different. The vesicle counts of all three fractions before incubation were significantly greater in the neonatal samples compared to those of the adults ($p < 0.005$). In both the adults and neonates, the top fraction had significantly more vesicles than the two lower fractions ($p < 0.01$ for both), but the middle and bottom fractions were not significantly different.

The individual fractions were incubated, and the results are shown in Table 3. The vesicle counts increased as the density of the fraction decreased in both the neonates ($p < 0.0005$) and the adults ($p < 0.002$). The neonatal red cells consistently acquired greater numbers of vesicles compared to the adult cells throughout the 144 h of incubation ($p < 0.005$). Vesicle formation in even the oldest neonatal fractions was greater than younger adult fractions containing higher reticulocyte counts.

The maintenance of incubating erythrocytes in a "physiologic" state was confirmed by demonstrating maintenance of normal levels of adenosine tri-, di-, and monophosphates, 2,3-diphosphoglycerate, and inorganic phosphates, using ^{31}P nuclear magnetic resonance spectroscopy throughout the 144-h *in vitro* in-

cubation. Fewer than 10% of incubating red cells demonstrated crenation, red cell loss (via hemolysis) was less than 3%, and the pH of the incubating media was maintained at 7.35–7.45.

Electron microscopic studies of incubated neonatal and adult erythrocytes demonstrated histologically indistinguishable intracellular vesicles in a minority of the incubated erythrocytes (Fig. 2).

The quantitation of Howell-Jolly bodies in several groups is shown in Table 4. These inclusions were demonstrable in only 30% of the neonates and did not differ significantly from the numbers found in the normal adults. There were significantly fewer Howell-Jolly bodies in the neonates compared to the children with functional hyposplenism matched for percentages of intraerythrocytic vesicles ($p < 0.01$) as well as to those with surgical asplenia ($p < 0.001$). The children with functional hyposplenism demonstrated a significant correlation between the number of Howell-Jolly bodies and the percentage of vesiculated erythrocytes ($r = 0.6$, $p = 0.009$), whereas the neonates did not ($r = 0.3$, $p = 0.2$).

Table 2. Increase in percentage of neonatal and adult erythrocytes containing vesicles during incubation *in vitro**

	Duration of incubation (h)		
	48	96	144
Neonates	9.3 ± 7.1	16.0 ± 6.5	19.2 ± 9.4
Adults	1.5 ± 2.1	2.6 ± 1.9	3.7 ± 1.2

* Vesicles were quantitated by determining the percentage of erythrocytes containing apparent surface pits with an interference contrast microscope at 1000X. Values expressed at each interval represent the percentage increase in erythrocytes with vesicles over the baseline value at the beginning of the incubation. Values are expressed as mean $\pm$ SD.

Table 3. Increases in percentage of density-separated erythrocytes containing vesicles during 144 h of *in vitro* incubation*

Neonate	
Upper fraction	37.6 ± 18.1
Middle fraction	14.6 ± 7.1
Lower fraction	12.2 ± 7.8
Adult	
Upper fraction	10.3 ± 6.0
Middle fraction	3.3 ± 2.2
Lower fraction	2.4 ± 2.0

* This is the percentage of erythrocytes that acquired intracellular vesicles during the incubation. It does not include the baseline percentage of vesiculated erythrocytes present before incubation.

Table 1. Characterization of unseparated and density-separated adult and neonatal erythrocytes before incubation

	% Reticulocytes	MCV (μ^3)	MCHC (g/dl)	G6PD (U/ml)	% Vesicles*
Neonates					
Unseparated blood	3.6	102.0	33.0	12.3	8.4 ± 5.0
Density-separated fractions					
Top fraction	23.1	104.9	32.5	16.1	19.3 ± 6.7
Middle fraction	3.7	101.2	32.9	14.1	8.2 ± 5.5
Bottom fraction	0.4	95.2	34.4	11.8	9.9 ± 5.6
Adults					
Unseparated blood	1.5	84.6	34.9	8.4	0.5 ± 0.5
Density-separated fractions					
Top fraction	10.5	84.7	32.6	9.5	5.4 ± 2.3
Middle fraction	1.2	83.8	33.0	7.7	1.3 ± 1.3
Bottom fraction	0.2	82.9	33.9	6.0	0.4 ± 0.5

* Percentage of erythrocytes containing any vesicles $\pm$ SD.

Fig. 2. A portion of a neonatal erythrocyte fixed after 144 h of *in vitro* incubation and then coated with cationized ferritin. The cell is examined using transmission electron microscopy at 40,000 $\times$. The inset contains the same vesicle at 120,000 $\times$. Note the heavy particulate coating of cationized ferritin on the exterior erythrocyte membrane. The vesicle is surrounded by bilayered membrane devoid of cationized ferritin, confirming its intracellular nature. The appearance of vesicles in neonatal cells was identical to those found in incubated adult erythrocytes.

Table 4. Quantitation of Howell-Jolly bodies

Group	No.	% Howell-Jolly bodies*	% Erythrocytes with vesicles†
Normal adults	10	0	0.5 $\pm$ 0.5
Normal neonates	20	0.005 $\pm$ 0.01	8.6 $\pm$ 5.3
Functional hyposplenism‡	20	0.03 $\pm$ 0.05	8.5 $\pm$ 5.4
Surgical asplenia	10	0.14 $\pm$ 0.02	25.3 $\pm$ 7.4

* Expressed as percentage $\pm$ SD of 10,000 erythrocytes containing any Howell-Jolly bodies.

† Expressed as percentage $\pm$ SD of 500 erythrocytes containing any vesicles.

‡ This group of 20 was selected from a group of 42 to provide as close a match of percentage of vesiculated erythrocytes as possible with the 20 normal neonates. Comparing these two groups with a *t* test gave a *t* = 0.08 and *p* > 0.9.

DISCUSSION

The evidence supporting the concept of neonatal hyposplenism is subject to question. Studies of splenic clearance in neonatal rats have produced conflicting results as to whether splenic function is impaired (26, 27) or normal (28, 29). Histologically, the spleen of the newborn rat and human demonstrate differences from the adult, but a relationship of these changes to any impairment of function has not been demonstrated (30–32).

Clinically, Heinz bodies (6, 33, 34) and Howell-Jolly bodies (3, 5) are signs of hyposplenism which are reported in neonates; however, both are not specific for hyposplenism (5) and are found in only a small minority of newborns (5–7). Because Howell-Jolly bodies are considered the better indicator of hyposplenism, we examined these inclusions. Finding Howell-Jolly bodies in only 30% of our neonates was surprising because almost all neonates have increased erythrocytic vesicles and are thereby assumed to be functionally hyposplenic. Neonates demonstrated fewer Howell-Jolly bodies than hyposplenic children with iden-

tical percentages of vesiculated erythrocytes. Newborns also failed to demonstrate the positive correlation of Howell-Jolly bodies and vesiculated erythrocytes as indicators of hyposplenism which was found in the hyposplenic children. These findings suggest that erythrocyte vesicles may not represent an accurate indicator of hyposplenism in neonates.

The most widely accepted method of evaluating splenic function is radionuclide scanning, which measures the spleen's ability to clear Tc^{99m} heat-damaged red cells or Tc^{99m} sulfur colloid. We could not ethically justify performing these studies on our subjects. However, several reports confirm that the neonatal spleen is able to clear both Tc^{99m} heat-damaged red cells and Tc^{99m} sulfur colloid as effectively as the spleen of adults (35–40). These observations in particular, along with the conflicting data presented earlier, fail to provide convincing evidence of functional impairment of the newborn spleen.

The single observation which appears to support strongly the concept of neonatal hyposplenism is the increase in erythrocytes containing intracellular vesicles. The assumption that this indicates hyposplenism is based upon experience with a wide variety of disorders in older children and adults in whom impaired splenic function was confirmed by other methodologies (2, 23, 41–43). It has been assumed that adult erythrocytes normally form these vesicles, which are then cleared by the spleen (15, 23). Our earlier studies (16) as well as our control adult incubation studies reported here demonstrate the capability of normal adult erythrocytes to form intracellular vesicles spontaneously that appear to be identical to those found in asplenic individuals. This is consistent with the assumption that these vesicles only accumulate *in vivo* as a consequence of diminished splenic clearance. Therefore, the finding of increased percentages of erythrocytes containing vesicles (3.9–24.3%) in three studies of newborns (7–9) led to the establishment of the concept of neonatal hyposplenism. It was hypothesized that the increased vesiculation of neonatal red blood cells is a result of a developmental phenomenon in which function of the immature spleen is impaired.

There is, however, an alternative explanation for the increase in erythrocytic vesicles in the newborn that is independent of splenic function. We suggest that the rate of formation of these inclusions is increased in the newborn and overcomes the otherwise normal capacity of the spleen to remove them. Neonatal red blood cells are known to undergo more readily a variety of endocytic processes (15), including receptor-mediated endocytosis (10–14) and drug-induced endocytosis (44). *In vivo* endocytosis throughout the life span of neonatal erythrocytes has been proposed as an explanation of some of the apparent differences between adult and neonatal red blood cells, including loss of surface membrane, diminished deformability, and higher sphingomyelin content (45).

Using our *in vitro* model to eliminate concomitant splenic clearance, neonatal erythrocytes acquired more intracellular vesicles than adult cells under as close to physiologic conditions as possible. The possibilities that increased vesicle formation was due simply to increased cell surface area or a greater proportion of reticulocytes in the neonates were considered; this seemed unlikely because the moderate increase in cell size (and surface area) as well as the modest increase in reticulocytes that are expected in neonates was unlikely to account for the 5-fold increase in vesicle formation *in vitro*. We suggest that the increase in erythrocytic vesicles in neonates is due to enhanced formation as opposed to impaired clearance due to hyposplenism. Unfortunately, our study does not exclude the possibility that an additional component of the increased vesiculation could be due to a concomitant impairment of splenic clearance. Experimental studies suggest that the spleen might be capable of removing a 5-fold increase in erythrocyte vesicles, but the applicability of these studies in adult dogs is unknown (46). However, considering our data, the weakness of the other data supporting impairment of splenic function in the neonate, and the normal ability of the neonatal spleen to clear sulfur-colloid and heat-damaged red cells, we suggest that the concept of neonatal hyposplenism should be challenged.

The incubation of density-separated erythrocytes further elucidates this process of vesicle formation. The efficacy of our density separation was confirmed by the reticulocyte counts, erythrocyte indices, and G6PD assays performed on density-separated samples (44, 45). Earlier studies of unincubated, density-separated neonatal erythrocytes noted vesicle formation to be most frequent in the denser older fractions and less frequent in the younger fractions (45). Conversely, our unincubated samples demonstrated higher percentages of vesicles in the youngest layers, although vesicle formation persisted in older layers. Our explanation for this discrepancy is that earlier studies only examined asplenic donors whose erythrocyte vesicles had already formed *in vivo*; even if younger cells form vesicles more rapidly, any additional vesicle formation with age will result in the older cells containing more total vesicles. In contrast, our incubation technique allows comparison of the actual rate of vesicle formation over a specific interval in various aged red cells, confirming that younger cells actually acquire vesicles most rapidly. It is also noted that because the percentage of cells acquiring vesicles was greater than the percentage of reticulocytes, this process is not limited to reticulocytes.

The process of vesicle formation is assumed to be endocytic in nature (16, 45). It may be a form of receptor-mediated endocytosis, which is a process limited mainly to neonatal reticulocytes (11, 15, 47). Further work is necessary to better define the nature of this process.

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