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Research Article

THE PHOSPHATE PARTITION OF THE ERYTHROCYTES OF NORMAL NEWBORN INFANTS AND OF INFANTS WITH HEMOLYTIC DISEASE

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The mammalian erythrocyte depends on a complex glycolytic mechanism for most of its energy. It is believed that this mechanism controls the transfer of cations across the cell membrane, and serves to maintain the biconcave shape of the red blood cell. Adenosine triphosphate and 2,3-diphosphoglycerate participate in the energy exchanges involved in these processes (1–9).

Alterations in the relationship of the phosphate fractions develop in erythrocytes during storage (10–16). Similar changes do not occur with the senescence of erythrocytes *in vivo* (12). The deviations from the normal pattern may be reversed following the transfusion of stored blood (14), and by the addition of some of the purine nucleosides *in vitro* (17–24). Abnormalities of the metabolism of the erythrocytes in hereditary spherocytosis, auto-immune hemolytic disease with spherocytosis, and in hereditary non-spherocytic hemolytic disease have been demonstrated (25–28). The literature contains only fragmentary data on partitioning of the acid-soluble phosphorus compounds of fetal erythrocytes (2, 29–32). Elevated values have been reported for the inorganic and ester phosphates in the red cells of normal infants (31, 32).

The purpose of this study was to find out if the action of specific antibodies on fetal erythrocytes produces any alteration in the phosphate partitioning. The intra-erythrocytic phosphate pattern of normal fetal red cells was studied and compared with that of the red cells of adults.

MATERIAL AND METHODS

All the infants studied had been delivered normally but were otherwise unselected. There were ten female and six male babies in the normal group and nine females and one male with erythroblastosis fetalis. The average weight of the normal infants was 7.6 pounds (6.1 to 9.0), and the weight of the infants with hemolytic disease averaged 6.8 pounds (5.1 to 8.4). The blood group of one normal infant was A and that of the mother B. The blood group of two infants with hemolytic disease was AB and their mothers were A_2 and B. The other infants were all products of homospecific pregnancies. The medication and anesthesia administered for delivery were similar in the two groups.

The cord blood [1] of the normal babies was drawn aseptically from the umbilical vein at the time of delivery. The blood of the infants with hemolytic disease was obtained at the time of the first replacement transfusion within the first eight hours of life. All of the blood specimens were collected into acid-citrate-dextrose solution (ACD) formula A[2] in the proportion of 100 ml. of blood to 15 ml. of preservative. The final concentration of glucose added was approximately 320 mg. per cent in whole blood. The specimens reached the laboratory within 30 minutes. Aliquots were distributed into sterile vacuum tubes. The chemical analysis of the fresh specimens was started immediately. The remaining aliquots were stored at 4° to 6° C. Mixing of the specimens during storage was avoided in order to duplicate the usual conditions under which "bank blood" is kept. Each specimen was cultured at the time of use, and the data were discarded if contamination was present.

For studying the effect of antibodies added *in vitro* the plasma was removed from a portion of the blood specimen and replaced with sterile anti-D serum [3] and ACD solution. This mixture and the control specimen were incubated at 37 ± 1° C in a water bath, and aliquots of each were distributed into sterile tubes for storage at 4° to 6° C.

Adenosine, 100 μM per ml. of 0.85 per cent sodium chloride, was added to a final concentration of 25 μM per ml. of packed red cells. An identical volume of 0.85 per cent sodium chloride was added to the control specimens. All the solutions were sterile.

A direct Coombs test was performed on each specimen containing antibodies.

Hematocrit determinations were done in duplicate using a high-speed microhematocrit centrifuge.[4]

For phosphate partition the red cells were washed three times with cold saline. The buffy coat was removed after each washing. The inorganic, total, 7-minute hydrolyzable, and 100-minute hydrolyzable phosphate

[1] Cord blood and fetal blood are used interchangeably.

[2] Trisodium citrate 2.20 per cent
Citric acid 0.80 per cent
Dextrose 2.45 per cent

[3] Native serum containing no diluent or preservative.

[4] International Co.

fractions were determined on cold, five per cent trichloracetic acid extracts of the washed, packed red cells by the method of Lowry and Lopez (33). The hydrolyses were conducted for 7 and 100 minutes in 1 N H_2SO_4 at 100° C. The total phosphate was determined after wet ashing the filtrate (34).

The phosphate fractions determined in this manner (12, 15, 35–38) are believed to represent: 1) inorganic phosphate; 2) labile phosphates released by 7-minute hydrolysis mainly from adenosine triphosphate (easily hydrolyzable); 3) phosphates split from adenosine monophosphate and hexose phosphates after 100-minute hydrolysis (difficultly hydrolyzable); 4) total phosphate; 5) largely the phosphates of 2,3-diphosphoglycerate which are estimated by subtracting the first three fractions from the total phosphate (non-hydrolyzable).

Duplicate determinations were performed throughout the study. Analysis of these data reveals:

Fraction*	Number of duplicate pairs	Standard deviation mg. % P	Percentage of values falling within 1 S.D.	3 S.D.
P_i	96	±0.2506	90.6	98.9
EH	94	±0.3965	86.1	100.0
DH	93	±0.5156	89.2	98.9
Total	94	±1.2153	52.1	93.6

* See legend, Table I.

P values were obtained from t calculated by the formula given by Snedecor (39) for groups of unequal sizes:

$$t = \bar{x}\sqrt{\frac{n_1 n_2 (n_1 + n_2 - 2)}{(n_1 + n_2) Sx^2}}$$

where

$\bar{x}$ = the difference between the group means.
Sx^2 = the pooled sum of the squares of the differences from the two group means.

RESULTS

Comparison of the erythrocytes of normal infants and adults

Trend during storage. The data presented in Table I indicate a greater increase in the inorganic phosphorus of the erythrocytes of normal cord blood than in the red cells of adults after storage for three weeks at 4° C. Simultaneously, the easily hydrolyzable and non-hydrolyzable phosphorus fractions of the fetal cells decreased significantly less, and the difficultly hydrolyzable phosphorus decreased appreciably more than in the erythrocytes of adults.

Average values. Comparison of the average values presented in Table I reveals that the inorganic phosphorus fraction (124 μM) of fresh erythrocytes from cord blood is significantly higher than the inorganic phosphorus level (65 μM) of the fresh cells of adults ($p < 0.001$), and the easily hydrolyzable phosphorus fraction in the erythrocytes of cord blood is significantly lower ($p = 0.02$). After storage the relationship of the inorganic phosphorus (934 μM) of the fetal cor-

TABLE I

Comparison of the changes during storage of the phosphate partition of erythrocytes from normal cord blood and from normal adults

Blood sample	Number of individuals	Days stored at 4° C	μM P/100 ml. cells P_i*	EH	DH	Non-H	Total
Normal cord	14	0	124† (*73–161*)	277 (*189–431*)	142 (*31–266*)	837 (*376–1,424*)	1,380 (*900–2,018*)
	10	21	934 (*734–1,372*)	225 (*185–288*)	41 (*0–138*)	350 (*88–588*)	1,550 (*1,066–1,861*)
Difference			+810	−52	−101	−487	+170
Normal adult	5	0	65 (*41–82*)	359 (*278–432*)	113 (*98–137*)	1,167 (*950–1,239*)	1,703 (*1,448–1,840*)
	7	21	692 (*632–767*)	218 (*198–264*)	82 (*28–156*)	437 (*377–547*)	1,430 (*1,324–1,500*)
Difference			+627	−141	−31	−730	−273
p values of differences			= 0.025	<0.001	= 0.01	= 0.005	>0.90

* P_i, EH, DH, and Non-H = inorganic, easily hydrolyzable, difficultly hydrolyzable, and non-hydrolyzable phosphate, respectively.
† Numbers not in parentheses represent means.
Numbers in parentheses represent ranges.

TABLE II

Statistical analysis of the differences in the changes in the intra-erythrocytic phosphate fractions which developed during storage of normal cord blood and the blood of infants with anti-D hemolytic disease *

Days stored at 4° C	P_i†	EH	DH	Non-H	Total
3	p‡ = 0.10	p = 0.70	p > 0.90	p > 0.10	p > 0.30
7	p > 0.05	p < 0.05	p = 0.025	p > 0.50	p = 0.50
14§	p < 0.005	p < 0.005	p < 0.01	p = 0.60	p > 0.80
21	p = 0.05	p < 0.001	p > 0.10	p > 0.30	p = 0.40

* Increase in P_i and decrease in EH and DH during storage was greater in the erythrocytes of infants with hemolytic disease. Variations in Non-H and Total phosphorus were not significant.
† See legend, Table I.
‡ p = probability value obtained from t. For calculation see methods section.
§ More completely presented in Table III.

puscles to that in the cells of adults (692 μM) remains similar ($p = 0.005$). Analysis of the remainder of the data reveals no significant differences.

Comparison of the intra-erythrocytic phosphate partitioning in normal newborn infants and infants with anti-D hemolytic disease

Trend during storage. The phosphate partitioning in the erythrocytes from aliquots of normal cord blood and of specimens obtained from infants with anti-D hemolytic disease was studied after storage at 4° C for 3, 7, 14 and 21 days. The trend of the changes in the phosphate fractions was observed to be similar after varying periods of storage. The inorganic phosphorus in the erythrocytes from infants with hemolytic disease rose significantly more rapidly than in the erythrocytes from normal cord blood. The easily hydrolyzable and difficultly hydrolyzable phosphate fractions of the cells of infants with hemolytic disease decreased more rapidly than in red cells from normal cord blood. The data of all these observations are not presented. The significance of the progressive differences in the phosphate partitioning which developed during storage of the red cells of the two groups of infants is presented in terms of probability values (p) in Table II. Representative data obtained after 14 days of storage at 4° C are given in more detail in Table III.

Average values. The only significant difference in the phosphate partition of the fresh erythrocytes of the two groups of infants is the higher level of

TABLE III

Comparison of the changes during storage of the phosphate partition of the erythrocytes from normal cord blood and infants with anti-D hemolytic disease

Blood sample	Number of infants	Days stored at 4° C	μM P/100 ml. cells				
			P_i*	EH	DH	Non-H	Total
Normal cord	14	0	124 (*73–161*)	277 (*189–431*)	142 (*31–266*)	837 (*376–1,424*)	1,380 (*900–2,018*)
	8	14	764 (*610–1,015*)	192 (*120–258*)	94 (*0–197*)	421 (*48–667*)	1,472 (*1,061–1,701*)
Difference			+640	−85	−48	−416	+92
Anti-D hemolytic disease	9	0	135 (*112–152*)	389 (*269–510*)	182 (*117–377*)	804 (*373–1,252*)	1,510 (*1,121–2,057*)
	7	14	1,039 (*874–1,181*)	156 (*11–284*)	43 (*0–104*)	440 (*176–747*)	1,677 (*1,441–1,939*)
Difference			+904	−233	−139	−364	+167
p values of differences			<0.005	<0.005	<0.01	= 0.60	>0.80

* See legend, Table I.

TABLE IV

Phosphate partition of normal group O, D positive adult and fetal erythrocytes exposed to anti-D serum in vitro *

Sample	Hours at 37° C	Days at 4° C following incubation	Direct Coombs	μM P/100 ml. cells				
				Pi†	EH	DH	Non-H	Total
Adult—O, D positive								
—unincubated control	0	0	—	116	358	72	1,124	1,670
—+ anti-D, unincubated	0	0	3+	104	292	125	1,002	1,523
—incubated control	3.5	0	—	302	312	108	542	1,263
—+ anti-D, incubated	3.5	0	3+	275	243	177	595	1,290
—stored control	0	21	—	632	198	156	514	1,500
—+ anti-D, stored	0	21	2+	634	200	225	565	1,623
Cord blood—O, D positive								
—unincubated control	0	0	—	131	288	141	745	1,305
—incubated control	21	0	—	639	290	0	764	1,682
—+ anti-D, incubated	21	0	4+	655	170	55	575	1,455
—stored control	0	14	—	820	176	197	505	1,701
—incubated, stored control	1	14	—	713	133	191	529	1,568
—+ anti-D, incubated, stored	1	14	4+	675	191	158	690	1,714

* Adult erythrocytes = erythrocytes of adults.
Fetal erythrocytes = erythrocytes from cord blood.
† See legend, Table I.

the easily hydrolyzable phosphorus found in the infants with hemolytic disease ($p < 0.005$). At 14 days the average inorganic phosphorus content of red cells from infants with hemolytic disease is significantly elevated above that in normal cord blood ($p < 0.005$). No significance can be assigned to the average values for the other phosphate fractions (Table III).

TABLE V

Regeneration of the phosphate partition with adenosine in anti-D hemolytic disease and in normal controls

Sample	Age of specimen at time of analysis	μM P/100 ml. cells				
		Pi*	EH	DH	Non-H	Total
Adult—control stored at 4° C for 25 days	25 days	688	131	117	624	1,562
—+ adenosine at 4° C after 21 days	25 days	39	368	226	924	1,558
Cord blood†—O, D negative						
—fresh control	0.5 hrs.	187	331	154	491	1,163
—incubated 23 hours at 37° C	23.5 hrs.	678	269	58	335	1,339
—+ adenosine at 37° C for 23 hours	23.5 hrs.	140	484	338	410	1,371
—stored 14 days at 4° C	14 days	752	320	57	377	1,506
—stored 14 days, incubated 1 hour at 37° C	14 days	644	302	304	152	1,402
—stored 14 days + adenosine at 37° C for 1 hour	14 days	253	441	333	359	1,386
Cord blood—A, D positive, anti-D hemolytic disease						
—control stored at 4° C for 20 days	20 days	1,150	488	19	381	2,037
—+ adenosine at 4° C after 16 days	20 days	613	424	121	330	1,488
Cord blood—O, D positive, anti-D hemolytic disease						
—fresh control	0.5 hrs.	195	1,351	0	23	1,570
—incubated 23 hours at 37° C	23.5 hrs.	1,203	53	67	295	1,619
—+ adenosine at 37° C for 23 hours	23.5 hrs.	638	207	221	669	1,735
—stored 14 days at 4° C	14 days	1,206	159	89	754	2,208
—stored 14 days, incubated 1 hour at 37° C	14 days	749	254	347	0	1,347
—stored 14 days + adenosine at 37° C for 1 hour	14 days	156	558	266	1,254	2,235

* See legend, Table I.
† Maternal anti-D titer 1:32 in albumin. History of three stillbirths. ABO groups of all infants compatible with mothers'.

TABLE VI

Phosphate partition of the erythrocytes in other forms of iso-immune hemolytic disease of the newborn

Sample	Age of specimen	Direct Coombs	μM P/100 ml. cells				
			P_I*	EH	DH	Non-H	Total
Anti-c hemolytic disease							
Infant 3 hrs. of age	fresh	3+	260	339	121	484	1,204
Blood stored at 4° C	20 days	3+	1,445	195	186	393	2,219
Anti-B hemolytic disease							
Infant 5 hrs. of age	fresh	(+)	149	230	245	435	1,059
Blood stored at 4° C	15 days	weak	999	286	129	422	1,835
Anti-A hemolytic disease							
Infant 18½ hrs. of age	fresh	1+	103	329	151	1,340	1,922
Blood stored at 4° C	14 days	(+)	765	362	168	230	1,525

* See legend, Table I.

The phosphate partition of the red cells of 11 normal infants and four with hemolytic disease was studied after incubation *in vitro* for one to 24 hours. The appearance of the alterations in phosphate partition was accelerated. The trend was toward higher inorganic phosphorus values in the infants with hemolytic disease, but the data are not sufficiently extensive to permit analysis.

Effect of anti-D antibodies added in vitro

Representative data from experiments in which the blood of D positive adults and infants was exposed to the action of incomplete (blocking) anti-D antibodies *in vitro* are shown in Table IV. Control studies were also performed using the cord blood of an Rh negative infant. Under the conditions employed, the action of the antibodies *in vitro* does not appear to have had any significant effect on the phosphate partitioning of the erythrocytes.

Reversal of the biochemical changes by adenosine

Adenosine was effective in reversing the alterations in phosphate partition produced by storage or incubation in the red cells of normal infants and infants with hemolytic disease. Representative data are given in Table V.

Observations in other varieties of iso-immune hemolytic disease

Phosphate partition studies of the red cells of infants with anti-c, anti-A, and anti-B hemolytic disease which were encountered during the period covered by this study are presented in Table VI. The blood specimens from these babies became available because they required treatment with replacement transfusions. The need for therapy became apparent in the infant with anti-A hemolytic disease because of rapidly deepening jaundice during the first day of life. The clinical and laboratory findings in the infant with anti-B hemolytic disease warranted transfusion treatment right after birth. In these two infants approximately 34 per cent and 83 per cent of the red cells appeared to be spherocytes (*i.e.*, cells having increased thickness /diameter ratio) in stained blood smears.

The increase in the easily hydrolyzable phosphorus after storage of the red cells of the infants with anti-A and anti-B hemolytic disease is an unexpected finding for which no suitable explanation can be given at present.

DISCUSSION

Many differences have been recorded between the erythrocytes of cord blood and those of adults. At the time of birth the red corpuscles are macrocytic and fetal hemoglobin predominates. At least a proportion of the fetal red cells at term have decreased osmotic and increased mechanical fragility (40). Sjölin (41) has shown that the erythrocytes from cord blood lose potassium more rapidly than those of adults when stored at 4° C. He believes that the structure of the fetal red cell membrane may be different. Hollingsworth (42) reported that the erythrocytes of cord blood have a shortened post-transfusion survival. The present study demonstrates that the phosphate partition of the red cells from fresh cord blood differs from that of the fresh red cells of adults. After storage

at 4° C the changes in the phosphate partition of the erythrocytes are not the same in cord blood as they are in the blood of adults (Table I).

It is possible that the deviations in the phosphate partition of fresh fetal cells are due to extrinsic factors such as the anoxia and acidosis at birth, and the anesthesia administered during labor. It is unlikely that the elevated leukocyte and reticulocyte counts present in the specimens used in these studies affected the phosphate partitioning in the erythrocytes to a significant degree. It has been demonstrated by others that leukocyte levels do not have any appreciable effect on the phosphate partition of erythrocytes (13). The same investigators reported higher values for the adenosine polyphosphates and 2,3-diphosphoglycerate in fresh reticulocyte-laden blood, but could not find any difference in the progression of the changes in phosphate partitioning during storage (13). The white cells degenerate during the storage of blood collected with ACD preservative, and do not contribute to the glycolytic activity of stored blood (15). The buffy layer of each specimen used in this study was removed when the specimens were washed.

The easily hydrolyzable phosphorus fraction in the fresh erythrocytes of infants with anti-D hemolytic disease was found to be higher than in normal infants (Table III). During storage the changes in phosphate partitioning in the erythrocytes of infants with hemolytic disease were found to differ quantitatively from those in normal infants (Tables II and III). This indicates that the glycolytic metabolism, in which the phosphate compounds play a major role, is probably altered in the erythrocytes which are coated by anti-D antibodies. The effect of adenosine in restoring the phosphate distribution in these cells toward normal parallels observations which have been reported in hereditary spherocytosis (25–27). The evidence that the reaction between the antigens and antibodies in the ABO blood group system is exothermic may have some bearing on the results of the experiments which have been presented (43). The attachment of anti-D antibodies to the receptors on fetal red cells has been assumed to be the most important factor in the production of this form of hemolytic disease. It remains to be shown why the clinical severity of the hemolytic disease is not regularly correlated with the height of the maternal titer or the degree to which the fetal erythrocytes are coated with antibodies (44). Previous attempts to solve this problem have not been fruitful. Morphologic changes in the red cells are not evident. Spherocytosis is rarely present in uncomplicated anti-D hemolytic disease, and the osmotic fragility is only slightly increased, whereas the mechanical fragility is within normal limits (40). Variations of complement level *in vivo* are probably not a determining factor (45). It is suggested that variations in the metabolic mechanisms, which determine the intracellular phosphate relationships, might supply the answer to this vexing problem. The limited number of patients studied precludes any conclusions at this time even though there seems to be a superficial correlation between clinical severity and the degree of upheaval of the intracorpuscular phosphate relationships. It is not unlikely that similar alterations in the intra-erythrocytic phosphate partition occur in other varieties of isoimmune hemolytic disease of the newborn.

SUMMARY

The phosphate partitioning of the erythrocytes of normal cord blood and of infants with hemolytic disease of the newborn has been investigated.

The inorganic phosphate fraction of fresh red cells from normal cord blood was found to be higher and the easily hydrolyzable phosphorus fraction lower than in the cells of adults. Significant differences in the alteration of the phosphate partitioning of these erythrocytes were demonstrated during storage at 4° C.

A higher level of easily hydrolyzable phosphorus was found initially in the erythrocytes of infants with anti-D hemolytic disease than in normal cord blood. During storage the changes in phosphate partitioning in the erythrocytes of the infants with hemolytic disease were found to differ from those in normal infants.

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