Erythrocyte Lipid Abnormalities in Reye's Syndrome

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ABSTRACT. Previous studies have demonstrated alterations in plasma free fatty acid content in Reye's syndrome (RS). We have therefore studied erythrocyte membrane lipids to determine if there are concomitant structural and functional modifications attributable to RS. Erythrocyte lipids were measured in children with RS and in critically ill children also requiring intensive care (ICU). In comatose RS patients erythrocyte phospholipid arachidonate was increased 2-fold relative to control ICU patients: 20.46 ± 2.14 versus $10.41 \pm 2.32\%$ of total erythrocyte phospholipid, $p < 0.05$. RS coma patients also demonstrated an increased ratio of erythrocyte phospholipid polyunsaturated/saturated fatty acids (0.76 ± 0.10) compared to ICU controls (0.48 ± 0.08 , $p < 0.05$). Erythrocyte cholesterol was higher in RS patients (79.00 ± 6.61 $\mu\text{g}/\text{mg}$ protein) than in ICU controls (59.74 ± 6.09 , $p < 0.05$). Erythrocyte malondialdehyde generation was decreased in comatose RS patients (404 ± 28 nmol malondialdehyde/g hemoglobin) versus ICU (517 ± 29 , $p < 0.05$). Although plasma vitamin E was depressed in RS patients, the erythrocyte vitamin E concentrations were no different in RS patients than in ICU patients. All RS patients had a typical viral prodrome and either a history of aspirin intake and/or measurable serum salicylate on admission. All of the biochemical abnormalities in RS patients listed above returned to values comparable to those of healthy RS siblings on recovery. The transient nature of these phenomena suggests that they were related to viral infection and/or aspirin rather than to intrinsic differences in lipid metabolism between RS patients and controls. (*Pediatr Res* 21: 352-356, 1987)

Abbreviations

RS, Reye's syndrome
PUFA, polyunsaturated fatty acids
RBC, erythrocyte
RBC^{-PL}, erythrocyte phospholipid
MDA, malondialdehyde
ICU, intensive care unit
SFA, saturated fatty acids
EC, erythrocyte cholesterol
PC, phosphatidyl choline
PE, phosphatidyl ethanolamine
PS, phosphatidyl serine
PL, phospholipid

RS is an encephalopathy occurring in children subsequent to a viral prodrome. Although the precise cause remains unknown despite two decades of study, there is evidence of a generalized disturbance of lipid metabolism. Pathologically there is "fatty degeneration of the viscera" with small lipid droplet accumulation in liver, brain capillary endothelial cells, kidney (loops of Henle), myocardium, and pancreatic acini (1). In RS patients studied at autopsy, hepatic triglycerides are markedly elevated whereas cholesterol and phospholipids are decreased; in brain, cholesterol is decreased (2).

In plasma of children with RS, free fatty acids are increased whereas triglycerides are decreased (3). Although earlier reports indicated that disproportionate elevation of the plasma concentration of short and medium-chain free fatty acids was characteristic of the free fatty acidemia in RS (4), recently Ogburn *et al.* (5) have demonstrated that the long-chain PUFA content of the free fatty acid fraction in RS serum is increased whereas the serum phospholipid PUFA are decreased. *In vitro* and *in vivo* experiments in animals have demonstrated that polyunsaturated fatty acids can cause cerebral edema (6-8), a cardinal clinical feature of RS. In a rabbit model of RS, Kang *et al.* (9) have shown that intracisternal injection of free PUFA leads to a reproducible dose-titratable encephalopathy; they and others have proposed that PUFA may be acting as toxins in RS.

There have been no attempts to measure the lipid profile of any tissue during acute RS. Since the majority of PUFA in the cells is in phospholipids, we believe tissue lipid analysis could add to the understanding of the role of PUFA in this syndrome. Hence, we chose to analyze RBC lipids, concentrating on the fatty acid composition of the phospholipids in RS patients in comparison to appropriate controls. Erythrocyte malondialdehyde generation after exposure of erythrocytes to H_2O_2 , an oxidant stress (10), was also measured as an aid in determining whether or not erythrocyte lipid peroxidation was occurring during RS. Vitamin E, the major lipid soluble antioxidant (11), was also measured in erythrocytes and plasma. In addition to the vitamin's role in protecting PUFA against peroxidation it may also stabilize membrane phospholipids (11) and inhibit phospholipase A_2 (12).

Erythrocytes were chosen for the present study because they are readily obtained in a noninvasive fashion and because they are thought to be a reasonable model for the lipids of plasma membranes in general (13). Furthermore, unlike other mammalian cells which can initiate phospholipid synthesis *de novo* (14), mature erythrocytes can alter phospholipid fatty acids only by deacylation of endogenous phospholipids and reacylation of the resulting lysophospholipids (15) and/or exchange of intact phospholipid molecules with exogenous phospholipids. Since erythrocytes cannot alter fatty acid chain length or the degree of unsaturation (16-20), RBC-PL composition during RS might provide a clue as to host tissue phospholipid composition prior

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to the onset of RS or, alternatively, RBC-PL might reflect acute changes in plasma.

Interestingly, RS patients differed from critically ill controls in the composition of erythrocyte phospholipid fatty acids, cholesterol, MDA generation and plasma vitamin E; these data are the subject of this report.

METHODS

Clinical material. The study was approved by the St. Louis University School of Medicine Institutional Review Board.

RS patients. Fourteen children with a diagnosis of RS were admitted to the study. Diagnosis was made according to criteria established by the Center for Disease Control (21) and staging was done according to the NIH consensus (22). More detailed clinical information is available on request. All RS patients had a typical viral prodrome and either a history of aspirin intake and/or measurable serum salicylate on admission. Patients were managed by careful monitoring and all received intravenous glucose (5–20% dextrose in a balanced electrolyte solution). In addition the following therapies were employed: intermittent intravenous mannitol ($\frac{1}{4}$ – $\frac{1}{2}$ g/kg) ($n = 13$), elective intubation and hyperventilation ($n = 10$), pentobarbital coma ($n = 4$), intracranial pressure monitoring ($n = 9$), and controlled hypothermia ($n = 2$). Mean age was 10.2 ± 1.2 yr. None of the patients received a blood transfusion during the study, and none experienced significant blood loss. In the first part of the study, which was conducted in patients 1–7, plasma vitamin E and neutral lipids were compared to values for healthy controls. The results obtained (see below) served as the motivation for performing the RBC studies described in patients 8–14 and for using critically ill children (ICU) patients as controls for the acute illness. Healthy RS siblings served as controls for the RS patients who were studied after full clinical recovery.

Controls. Healthy Children. These were five healthy children whose blood was obtained after an overnight fast. The mean age was 10.8 ± 1.0 yr.

ICU Patients. The next patient admitted to the pediatric ICU of the same sex and age ± 3 yr as the RS patient served as a control for the acute illness. The mean age was 11.7 ± 0.7 yr. Diagnosis in the five ICU controls included liver laceration, open heart surgery, varicella encephalitis, hemolytic uremic syndrome, and Guillain-Barré syndrome.

RS Siblings. These siblings were six healthy children whose blood was obtained after an overnight fast. The mean age was 11.0 ± 0.8 yr.

Sample collection. Blood was collected in EDTA containing tubes and placed on ice immediately. Plasma was separated and stored at -70°C until analysis. Erythrocyte MDA production was studied within 24 h of sample collection as we determined that the assay was stable at 4°C for that length of time. For analysis of vitamin E, phospholipid, and cholesterol, cells were washed three times with 10 volumes of normal saline and then stored at -70°C until analysis.

In the patients, blood samples were drawn on admission, periodically during the acute illness, and on full clinical recovery; a single sample was obtained from each of the controls.

Laboratory studies. Plasma. Vitamin E was measured in all RS patients and control groups according to the method of Martinek (23). Cholesterol and triglycerides (neutral lipids) were measured in RS patients 1–7 and in healthy children according to Christensen *et al.* (24). Vitamin E data were expressed as the ratio of vitamin E to neutral lipids. Plasma salicylate was measured on admission (25).

Erythrocytes. These studies were performed in RS patients 8–14, in ICU controls, and RS siblings. MDA production by erythrocytes incubated in H_2O_2 was measured by the method of Bidder and Jaeger (10). Vitamin E (α -tocopherol) was measured by high-pressure liquid chromatography (26) and expressed on the basis of total lipid (27). Lipids were extracted and analyzed

as follows: 0.8 ml of RBC suspension were hypotonically lysed with 10 ml 5 mM Tris buffer pH 7.4 and centrifuged at $35,000 \times g$ at 4°C for 30 min. The pellet was resuspended in 10 ml fresh buffer and centrifuged as above. Then the pellet was resuspended in 500 μl buffer. An aliquot was taken for protein analysis (28). Four hundred μl of the membrane homogenate were added to 5 ml chloroform:methanol [2:1] (29). The chloroform:methanol extract was then spotted on a thin layer chromatography Silica Gel G plate (Merck, Scientific Products) and developed in the solvent system of petroleum ether:ether:acetic acid (75:25:1). Since free fatty acids (<20 ng/mg protein), triglycerides (<80 ng/mg protein), and cholesterol esters (<40 ng/mg protein) were not present in appreciable amounts, these were not analyzed further. For phospholipid fatty acid analysis, silica gel spots were extracted with 500 μl chloroform to which was added 600 μl 0.5 N NaOH in methanol and 2 μg 21:0 fatty acid as internal standard. After 10 min 600 μl 6 N HCl were added and samples were centrifuged $900 \times g$ for 5 min. The organic phase containing fatty acid methyl esters was dried under nitrogen and resuspended in 10 μl heptane. Subsequently an aliquot (2- μl) was injected into a gas liquid chromatography column (10% SP 2330 on 100/120 Chromasorb WAW). Fatty acid methyl esters were analyzed by flame ionization on a Hewlett Packard 5830A gas chromatograph. The data were expressed as relative area percent (area of individual fatty acid/area of total fatty acids $\times 100$). Cholesterol was measured by the method of Lange and Ramos (30).

Statistics. Mann-Whitney two sample test was used. Data are reported as mean $\pm$ SE. P values < 0.05 were considered to be significant.

RESULTS

Plasma studies. In patients 1–7, plasma vitamin E was markedly lower than that of healthy controls (Fig. 1a) during the acute illness but returned to control values on recovery without administration of supplemental vitamin E to the patients. Plasma neutral lipids (triglycerides plus cholesterol) were also depressed in the patients (Fig. 1b) in comparison to the healthy children; however, the ratio of plasma vitamin E:neutral lipids was lower in RS patients than controls (Fig. 1c). Values for the RS patients were comparable to those of healthy children on recovery.

These results served as the rationale for the RBC studies described below in RS patients 8–14 and for using critically ill children as controls for the acute illness since it was likely that the nutritional status of the RS patients was more comparable to that of the ICU patients than it was to that of healthy children. Plasma vitamin E was also measured in RS patients 8–14. In Figure 2 plasma vitamin E is shown for this second group of RS patients. Low values very similar to those found in RS patients 1–7 were observed (significantly lower than ICU controls only at 24 h). Values for ICU controls in turn were decreased compared to healthy RS siblings (Fig. 2).

Erythrocyte studies. There were several notable differences in RBC phospholipid fatty acids between RS patients (nos. 8–14) and ICU controls during the first 48 h of hospital admission. In Figure 3, total RBC phospholipid PUFA, consisting of 18:2 + 18:3 + 20:3 + 20:4 + 22:4 + 22:5 + 22:6, and total RBC phospholipid SFA, consisting of 14:0 + 16:0 + 18:0, are shown for the various groups. In the RS patients who progressed to NIH stage III–IV coma ("RS coma"), PUFA were increased ($p < 0.05$) while SFA were decreased ($p < 0.05$) as compared to ICU controls; consequently the ratio of PUFA/SFA was about twice as high in comatose RS patients as in ICU controls ($p < 0.05$). Total SFA was also lower in the whole group of RS patients compared to ICU controls ($p < 0.01$). For recovered RS patients *versus* siblings, values for PUFA were 28.06 ± 3.40 *versus* $26.90 \pm 4.21\%$ of total RBC phospholipids; for SFA were 39.81 ± 2.26 *versus* $38.42 \pm 2.32\%$; and for PUFA/SFA were 0.77 ± 0.13 *versus* 0.76 ± 0.06 ; none of these comparisons was significant.

Fig. 1. Time course of plasma vitamin E and neutral lipids in Reye's patients (nos. 1-7) versus healthy children. Values are represented as mean $\pm$ SE. RS versus healthy controls $*p < 0.05$, $***p < 0.001$.

Table 1 provides values for individual erythrocyte phospholipid fatty acids. The fatty acids of most interest were 16:0, 18:2, and 20:4. Compared to ICU controls, values for 16:0 were lower for RS patients ($p < 0.05$) whereas those for 18:2 were higher ($p < 0.05$). The amount of 20:4 was also higher in comatose RS patients than ICU ($p < 0.05$), RS sibs ($p < 0.05$), and RS recovery ($p < 0.03$). RS recovery values for all of the major individual erythrocyte phospholipid fatty acids mentioned above (with the exception of 16:0) did not differ from those of RS sibs.

RBC cholesterol in RS patients was 79.00 ± 6.61 $\mu\text{g}/\text{mg}$ protein, which was higher than that of ICU controls (59.74 ± 6.09 , $p < 0.05$) but lower than that of RS sibs (98.93 ± 6.73 , $p < 0.05$). ICU values were lower than sibs ($p < 0.004$). However, RS recovery values of cholesterol (65.88 ± 9.53 $\mu\text{g}/\text{mg}$ protein) did not differ significantly from sibs ($p < 0.06$).

Erythrocyte MDA values in comatose RS patients were lower on admission (404 ± 28 nmol MDA/g hemoglobin, $p < 0.05$) and days 2-3 (371 ± 54 , $p < 0.05$) than ICU patients (517 ± 29). On the other hand, ICU values were higher than RS sibs ($430 \pm$

28 nmol MDA/g hemoglobin, $p < 0.04$). RS recovery values were similar to those of sibs.

Despite the fact that plasma tocopherol in RS patients during acute illness was lower than values in other groups, RBC tocopherol in the patients (1.09 ± 0.42 $\mu\text{g}/\text{mg}$ lipid) did not differ significantly from ICU (0.101 ± 0.02 , $p < 0.07$) or sibs (0.167 ± 0.37) because of the wide range of values. Of interest is that three of the RS patients, two of whom were in the coma group, had values as high as 1.69, 1.63, and 2.59 μg tocopherol/mg lipid, which were 10-20 times as high as in any of the children from the ICU or sib groups. RS recovery values (0.51 ± 0.32 μg tocopherol/mg lipid, $p < 0.05$) were higher than those of ICU ($p < 0.05$).

DISCUSSION

The decrease in plasma vitamin E in the RS patients may be secondary to several factors. The least likely is vitamin E malabsorption since the patients were well-grown children whose plasma vitamin E returned to normal on recovery without dietary vitamin E supplementation. Decreased vitamin E intake may be a partial explanation for the lower values observed in RS patients and probably does account for the decreased plasma vitamin E in ICU patients (31). The hypolipidemia characteristic of RS patients was probably responsible for the somewhat lower plasma vitamin E values in RS patients compared to ICU controls. An alternative explanation is that the plasma vitamin E may be low in RS patients because of transfer of the vitamin from the plasma

Fig. 2. Time course of plasma vitamin E in RS patients (nos. 8-14) in comparison to ICU and RS sibling controls. Values are represented as mean $\pm$ SE. RS versus ICU $*p < 0.05$. ICU controls were lower than RS sibs $p < 0.05$.

Fig. 3. Relative percent of erythrocyte phospholipid fatty acids in RS patients and ICU controls. RS versus ICU $*p < 0.05$, $**p < 0.01$. Values for RS patients are mean $\pm$ SE of two to three samples obtained from each patient during first 48 h of hospital admission. Values in all groups are represented as mean $\pm$ SE.

Table 1. Major erythrocyte phospholipid fatty acids (relative area percent)

	14:0	16:0	18:0	18:1	18:2	18:3	20:3	20:4	22:4	22:5	22:6
RS (acute) (n = 7)	0.33 ±0.12	20.95* ±1.23	19.60 ±1.36	23.38 ±0.79	11.53* ±0.82	0.05 ±0.03	0.92 ±0.36	15.76 ±2.40	2.04 ±0.72	0.10 ±0.09	0.26 ±0.18
RS coma (n = 3)	0.20 ±0.08	19.13* ±1.20	19.07 ±1.87	23.97 ±1.38	12.70 ±0.94	0.04 ±0.03	0.63 ±0.28	20.46* ±2.14	2.96 ±1.19	0.00 ±0.07	0.08 ±0.07
ICU (n = 4)	0.22 ±0.09	25.76 ±1.4	22.01 ±0.57	25.29 ±1.9	10.03 ±0.50	0.0	0.40 ±0.18	10.41 ±2.32	1.08 ±0.55	0.18 ±0.11	0.29 ±0.19
RS sibs (n = 6)	0.35 ±0.10	18.99 ±0.95	19.13 ±1.48	20.09 ±1.35	10.05 ±0.66	0.10 ±0.05	0.89 ±0.27	11.54 ±1.96	2.27 ±1.13	1.08 ±0.51	1.56 ±0.65
RS (recovery) (n = 6)	0.21 ±0.19	21.93† ±1.91	19.39 ±1.77	23.59 ±1.99	12.05 ±1.42	0.05 ±0.09	0.73 ±0.35	12.47 ±1.44	1.69 ±0.53	0.19 ±0.18	0.56 ±0.31

* RS vs ICU: $p < 0.05$.† RS recovery vs RS sibs: $p < 0.05$.

to some lipid rich compartments of the body. Although it was originally thought that there was a positive correlation between plasma and RBC tocopherol (32), Bieri *et al.* (33) recently demonstrated that induction of hypolipidemia in rats resulted in increased erythrocyte tocopherol independent of dietary vitamin E; some of our RS patients appeared to demonstrate this phenomenon.

The lower RBC MDA values in the comatose RS patients compared to ICU controls are probably explained by the increased amounts of the free-radical scavengers, cholesterol (34), and/or vitamin E in the RBCs of those patients.

RBCs do not have *de novo* synthesis of cholesterol from acetate since they lack HMG CoA reductase (35); thus uptake of cholesterol from plasma is the major determinant of EC (36). Factors known to increase EC include liver disease and decreased plasma lecithin:cholesterol acyltransferase (36). Factors that decrease EC include fasting and increased RBC age (36). Prolonged fasting probably accounts for the decreased EC values in both the ICU and acutely ill RS patients.

In order to interpret our findings of altered RBC-PL fatty acids in RS patients, it is first pertinent to review determinants of RBC-PL fatty acids in healthy man. Exogenous factors known to be of influence include diet, disease, red blood cell age, and the plasma lipid profile. Farquhar and Ahrens (37) showed that in man the linoleic (18:2) and oleic (18:1) contents of RBC-PLs could be influenced by the quantity of these fatty acids in the diet, the changes occurring after ingestion of the diet for about 4–6 wk. Diseases in which RBC fatty acids are abnormal have been reviewed by Nelson (38); however, none has the abnormalities of increased PUFA observed in our RS patients.

The importance of plasma in influencing RBC-PL content was recognized almost two decades ago. The increased serum PUFA in the free fatty acid fraction known to occur in RS (5) may account for some of the increased RBC-PL PUFA which we observed in our patients. RBC-PL classes also influence the RBC-PL fatty acid profile since each has a characteristic distribution of fatty acids (39). For example, the increased 18:2 observed in RS RBC-PL could reflect a change in RBC PC (present in the outer sides of the RBC membranes) since 18:2 is more abundant in PC than in other RBC-PL classes (39).

The observed increase in the proportion of RBC-PL PUFA in the comatose RS patients in our study appears to be due primarily to increased RBC-PL 20:4. Of the factors described above which influence RBC-PL fatty acids, those known to decrease RBC 20:4 include liver disease (40), protein-calorie malnutrition (41), biotin deficiency (42), acrodermatitis enteropathica (43), and increased red blood cell age (38). Factors known to increase RBC 20:4 include the newborn state (44) and increased plasma arachidonate. In the study of Ogburn *et al.* (5), however, serum free arachidonate was not significantly increased in RS patients during the acute illness ($4.5 \pm 3.9 \mu\text{g/ml}$) compared to recovery (2.4 ± 2.0). The bulk of RBC-PL arachidonic acid is distributed

in PE and PS, both of which are located in the inner RBC membrane (39). In Reed's (18) study of PL exchange between plasma and RBCs there was no metabolic turnover of PS; thus the possibility exists that PE would be the phospholipid most likely to be altered in erythrocytes of patients with RS.

Aspirin has been linked epidemiologically to RS (45), and each of our patients had either a history of aspirin intake, detectable serum salicylate or both. The role of aspirin, if any, in the abnormalities of plasma vitamin E and RBC lipids observed in our RS patients is conjectural. It is possible that the effect of aspirin in inhibiting prostaglandin synthetase (46) in a variety of tissues (the enzyme is not thought to be present in RBCs) results in increased concentrations of 20:4 in plasma during the period of aspirin intake, and secondarily, in the erythrocytes. Aspirin could also increase RBC-PL 20:4 by increasing the acylation of this fatty acid to lysophospholipids (47).

The transient nature of the abnormalities in erythrocyte lipids and plasma vitamin E observed during acute RS suggests that children who develop RS do not differ intrinsically in lipid metabolism from their healthy siblings. Studies in humans have shown that neither influenza virus infection alone (48) nor aspirin alone (49) has any effect on serum triglycerides and total cholesterol. Further, influenza virus infection has no effect on serum free fatty acids and phospholipids (48), whereas aspirin may decrease serum free fatty acids (50). The contrast between the abnormalities in plasma as well as RBC lipid composition in our patients (all of whom had taken aspirin during a viral infection) and the absence of a RS-like pattern in serum lipids with virus infection alone or aspirin intake alone suggests that study of synergistic effects between these two factors on lipid metabolism will be important in RS research. The erythrocyte arachidonate abnormality that we observed in the RS patients, especially when taken in the context of a recent report of lipoxigenase pathway in erythrocytes for metabolism of arachidonic acid (51), merits further investigation in RS patients and animal models of the syndrome.

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