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Hemolytic Anemia of Owl Monkeys: Red Blood Cell Lipid Alterations[1] (41340)

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Abstract. The owl monkey (*Aotus trivirgatus*), a species of New World monkey represented by several different karyotypes, develops hemolytic anemia in captivity. Susceptibility to anemia varies as a function of karyotype, and the hemolytic process responds to vitamin E. Since the plasma concentration of vitamin E in these owl monkeys was normal, it was hypothesized that the anemia may reflect a lipid abnormality in the red blood cell (RBC) membrane which was stabilized by supplemental tocopherol and that the basic RBC lipids would differ as a function of owl monkey karyotype. To test this hypothesis the RBC membrane cholesterol, phospholipid, fatty acids, and free cholesterol to phospholipid ratio (FC/PL) were compared between (a) anemic and nonanemic owl monkeys, (b) owl monkeys of different karyotypes, and (c) between different primate species. As a species, the owl monkey exhibited a unique RBC phospholipid profile, having reduced proportions of phosphatidylserine (PS) and phosphatidylethanolamine (PE) and more phosphatidylcholine (PC) and sphingomyelin (Sph) when compared to other primates, including man. As a function of karyotype, owl monkeys of karyotype VI (anemia resistant) had a normal polyunsaturated fatty acid (PUFA) profile in RBC phospholipids and less RBC membrane cholesterol contributing to a normal FC/PL ratio, whereas RBCs from susceptible and overtly anemic owl monkeys revealed low levels of PUFA and increased RBC membrane cholesterol that led to an elevated FC/PL ratio. The etiology of these lipid alterations and their association with anemia remains to be elucidated.

The captive owl monkey (*Aotus trivirgatus*) is prone to develop hemolytic anemia which can be ameliorated by an injection of vitamin E and selenium (1). However, plasma vitamin E as well as red blood cell (RBC) glutathione peroxidase, glutathione reductase, glucose-6-phosphate dehydrogenase, and 6-phosphogluconate dehydrogenase levels are within the range of normal values reported for most mammalian species (1), suggesting that antioxidant treatment is supportive, but probably not the etiologic factor underlying the pathogenesis of the disease. Furthermore, among the several karyotypes available for study at the New England Regional Primate Research Center, karyotype VI has been found to be resistant to the anemia (2).

Since vitamin E is thought to stabilize the polyunsaturated fatty acids (PUFA) of phospholipids and thereby play a major role in overall membrane stability, it was hypothesized that hereditary or environmental factors, including nutrition (such as inadequate dietary essential fatty acids), had altered the lipid composition of the RBC membrane in susceptible karyotypes of owl monkeys rendering it vulnerable to oxidative hemolysis (3, 4). To explore these possibilities, the RBC membrane cholesterol and phospholipid profile, fatty acid composition, and free cholesterol to phospholipid (FC/PL) ratio of anemic and nonanemic owl monkeys were compared to RBCs of other primate species maintained under similar conditions.

The results obtained suggest that anemia in owl monkeys is associated with three important changes in the RBC membrane: an

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increase in RBC cholesterol content leading to an elevated FC/PL ratio, an altered phospholipid profile, and an abnormal distribution of phospholipid fatty acids.

Materials and Methods. *Monkeys.* The 53 owl monkeys, representing seven karyotypes, were part of the 150 *Aotus* spp. maintained at the New England Regional Primate Research Center (NERPRC) for the past 12 years. They were fed high-protein Purina Monkey Chow and represented both sexes between the ages of 5 and 12 years. Blood samples were also obtained from seven cotton-top marmosets of both sexes maintained on Purina Chow. The 4 cebus, 4 squirrel, and 14 rhesus monkeys were maintained in the Nutrition Department of the Harvard School of Public Health and fed Purina Chow, except for 6 of the rhesus monkeys which were fed semipurified diets containing corn oil or coconut oil and 0.1% cholesterol (5).

Hematological evaluation. Data relating to the nature and degree of anemia have been described previously (1, 2). In this investigation, anemia refers to monkeys with hemoglobin values less than 10 g/dl and with circulating nucleated RBCs in their peripheral blood smear. Nonanemic monkeys had no circulating nucleated RBCs and hemoglobin values ranged from 14 to 16 g/dl.

Phospholipid determination. For RBC lipid analysis, whole blood was collected into tubes containing EDTA and centrifuged at 2000 *g* for 30 min at 4°. The RBCs were washed three times in 0.9% saline and an aliquot hemolyzed in 1 ml distilled water. Lipids from the RBCs were extracted in isopropanol (6) and chloroform ($CHCl_3$) followed by filtration of the extract through Whatman No. 2 filter paper. To reduce the possibility of oxidation, 0.02% butylated hydroxy toluene (BHT) was added to the isopropanol and $CHCl_3$, and the lipid extractions performed on ice and under nitrogen. Phospholipid classes were separated by two-dimensional thin-layer chromatography using silica gel H (Merck) plates impregnated with magnesium acetate. Following development in the solvent systems containing (i) $CHCl_3$:MeOH:NH_4OH (13:5:1) and (ii) $CHCL_3$:acetone:MeOH:acetic acid:water (6:8:2:2:1) (7), the phospholipids were visualized along with appropriate standards in iodine vapor, scraped, and assayed for phosphorus by the procedure of Bartlett (8).

Cholesterol determination. Cholesterol was assayed by the method described by Wybenga *et al.* (9).

Fatty acid determination. Fatty acid methyl esters of the total lipid extract were prepared with methanolic HCl (10) and analyzed with a Perkin–Elmer Model 3920 gas chromatograph (Perkin–Elmer Corp., Norwalk, Conn.) equipped with a flame ionization detector and 8-ft stainless steel columns containing 10% Silar 10C in Gas Chrom Q (100/200 mesh).

Results. *Cholesterol and phospholipid.* RBCs from nonanemic but susceptible owl monkeys of karyotypes II and III contained more cholesterol than anemia-resistant karyotype VI owl monkeys. The RBC cholesterol content was even higher in overtly anemic owl monkeys compared to nonanemic monkeys independent of karyotype. The total phospholipid RBC content of anemic owl monkeys was also slightly higher than nonanemic animals independent of karyotype (Table I).

While no differences were observed in the overall RBC phospholipid profile for most primates, including humans, the owl monkey differed appreciably from the others (Table II). Owl monkeys, independent of karyotype, had a significantly smaller percentage of PS and PE and more PC and Sph than the other primate species. The latter did not differ between each other and were similar to human RBCs with the exception of a higher percentage of Sph in human RBC (Table II). No differences in the distribution of owl monkey RBC phospholipids were evident between various karyotypes or between anemic and nonanemic monkeys (Table III).

The FC/PL molar ratio of RBC membranes from anemia-resistant, karyotype VI owl monkeys (1.02 ± 0.08, mean ± SD) was similar to rhesus monkeys (1.04 ± 0.20) and normal values for humans (0.97) (11) (Table I). By contrast, susceptible nonanemic owl monkeys of karyotypes II and III had elevated FC/PL ratios (1.35 ± 0.20 and 1.24 ±

TABLE I. ERYTHROCYTE CHOLESTEROL AND PHOSPHOLIPID CONCENTRATION IN PRIMATES

Species	N	μmole/10^{10} cells		Cholesterol/phospholipid (μmole/μmole)
		Cholesterol	Phospholipid	
Owl monkey				
Nonanemic				
Karyotype II	18	2.85 ± 0.54[a]	2.14 ± 0.39	1.35 ± 0.20
Karyotype III	18	2.65 ± 0.34	2.20 ± 0.44	1.24 ± 0.27
Karyotype VI	12	2.08 ± 0.18	2.04 ± 0.12	1.02 ± 0.08
Anemic				
Karyotypes II–IV	5	3.85 ± 0.46	2.63 ± 0.26	1.47 ± 0.10
Squirrel monkey	3	3.12 ± 0.22	2.21 ± 0.21	1.42 ± 0.14
Marmoset monkey	3	2.77 ± 0.15	2.06 ± 0.10	1.36 ± 0.03
Rhesus monkey[b]	14	2.98 ± 0.31	2.90 ± 0.19	1.04 ± 0.20
Human[c]	10	2.90 ± 0.30	3.00 ± 0.40	0.97

[a] Mean ± SD.
[b] Eight of the rhesus were fed Purina Chow; six were fed fat and cholesterol modified diets (see text).
[c] Ref. (11).

0.27, respectively), which tended to be even higher (1.47 ± 0.10) in overtly anemic owl monkeys. However, the RBCs from squirrel monkeys and marmosets had relatively high FC/PL ratios (1.42 ± 0.14 and 1.36 ± 0.03, respectively), which were similar to susceptible and anemic owl monkeys.

Fatty acids. Comparison of RBC phospholipid fatty acids from anemic, nonanemic, and resistant (karyotype VI) owl monkeys indicated that the latter had significantly more PUFA and less palmitic acid than either the anemic or susceptible karyotypes (Table IV). The fatty acid profiles of squirrel monkeys and marmosets were similar to the karyotype VI owl monkeys. The ratio of PUFA to saturated fatty acids (SFA) from RBC phospholipids of karyotype VI monkeys (0.76 ± 0.17) was significantly higher than that in the other owl monkey karyotypes (0.21 ± 0.19), but similar to that of humans (0.77 ± 0.15).

Discussion. The hypothesis that an altered lipid composition of the owl monkey RBC membrane might be associated with susceptibility to the hemolytic anemia in that species is supported by three observations. First, as a species, owl monkeys had significantly less PE and PS in their RBCs when compared to other primate species; second, RBCs from susceptible and anemic owl monkeys had elevated FC/PL molar ratios; and third, the RBC phospholipids in all susceptible and anemic owl monkeys revealed a striking PUFA deficit, particularly

TABLE II. PERCENTAGE DISTRIBUTION OF RED BLOOD CELL PHOSPHOLIPIDS IN FIVE SPECIES OF MONKEYS AND MAN

Species	N	PE[a]	PC	PI	PS	Sph
Owl[b]	31	19.3 ± 3.4[c]	45.6 ± 4.3	1.9 ± 1.4	2.1 ± 1.7	31.0 ± 4.8
Cebus	4	33.6 ± 1.6	31.1 ± 1.5	1.9 ± 0.2	15.4 ± 0.3	18.0 ± 2.3
Marmoset	7	35.6 ± 2.0	33.2 ± 2.7	3.3 ± 1.5	14.6 ± 1.0	13.4 ± 2.9
Squirrel	4	31.4 ± 2.2	40.6 ± 1.6	1.3 ± 0.6	10.1 ± 1.8	16.5 ± 3.0
Rhesus[d]	9	32.0 ± 3.0	41.2 ± 3.4	2.3 ± 0.9	11.5 ± 1.8	13.3 ± 3.2
Human[e]	9	29.4 ± 0.8	30.4 ± 1.3	12.4 ± 1.2	(PI + PS)	27.7 ± 1.2

[a] Phosphatidylethanolamine (PE), phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylserine (PS), sphingomyelin (Sph).
[b] Nonanemic owl monkeys (all karyotypes).
[c] Mean ± SD.
[d] Three of the rhesus were fed Purina Chow; six were fed fat and cholesterol modified diets (see text).
[e] Ref. (18).

TABLE III. PERCENTAGE DISTRIBUTION OF RED BLOOD CELL PHOSPHOLIPIDS FROM ANEMIC OWL MONKEYS AND NONANEMIC OWL MONKEYS ACCORDING TO KARYOTYPE

Karyotype	*N*	PE	PC	PI	PS	Sph
Nonanemic						
I	6	19.9 ± 1.8[a]	45.8 ± 2.8	2.2 ± 1.1	1.1 ± 0.9	30.8 ± 2.7
II	11	16.7 ± 1.5	45.4 ± 4.6	1.5 ± 1.3	2.3 ± 1.5	34.1 ± 5.5
III	8	22.2 ± 4.5	46.1 ± 5.7	2.2 ± 2.2	2.0 ± 2.0	27.4 ± 3.7
IV, V, VII	3	19.3 ± 3.6	46.9 ± 3.0	1.8 ± 0.8	3.2 ± 2.5	28.9 ± 2.4
VI	3	19.5 ± 1.3	43.6 ± 4.0	1.8 ± 0.5	3.2 ± 1.8	31.9 ± 2.2
Anemic						
III	5	19.3 ± 1.5	49.7 ± 4.5	2.7 ± 1.4	0.6 ± 0.6	27.8 ± 4.8

[a] Mean ± SD.

in arachidonic acid. Anemia in owl monkeys occurs in association with all three abnormalities.

Each of these three factors presumably influences the hemolytic process since the rigidity and fluidity of the RBC membrane is determined to a large extent by the membrane cholesterol content and the fatty acids present in the phospholipids (11–15). Increasing fatty acid unsaturation enhances fluidity, permeability, and flexibility because the fatty acids are unable to pack as closely as they do when saturated (16). Unsaturation may also favor the formation of phospholipid micelles in the membrane which, in turn, increases permeability (17, 18). Increasing methylation of PE to form PC also reduces the microviscosity of the membrane (19). On the other hand, cholesterol increases rigidity and decreases fluidity by limiting fatty acid chain movement (20).

Membrane phospholipids. The low PE and PS concentrations characterizing the RBC phospholipid profile of the owl monkey are suggestive of a peroxidative hemolysis. The PE and PC contained the highest percentages of PUFA, i.e., 31 and 35%, respectively. Since the PE molecule resides primarily on the cytoplasmic surface of the membrane (21), it would be exposed to intracellular peroxides. However, RBC intracytoplasmic peroxides generally are metabolized by the glutathione peroxidase system, which appears to be intact in owl monkeys (1). In addition, the therapeutic effect of vitamin E on the hemolytic process (1) suggested that peroxidation of PUFA in RBC phospholipids may have occurred. The RBCs from infants (22–24) and sheep (25) suffering from the peroxidative hemolysis of vitamin E deficiency undergo a significant reduction of PE from approxi-

TABLE IV. PERCENTAGE DISTRIBUTION OF RED BLOOD CELL PHOSPHOLIPID FATTY ACIDS IN MONKEYS AND MAN

Species	*N*	$C_{16:0}$[a]	$C_{18:0}$	$C_{18:1}$	$C_{18:2}$	$C_{20:4}$	Other
Owl monkey							
Nonanemic							
Karyotype II	5	40.4 ± 8.2[b]	16.2 ± 3.1	23.1 ± 5.2	11.9 ± 0.3	4.1 ± 3.6	4.3 ± 1.8
Karyotype III	4	43.9 ± 0.7	18.0 ± 2.7	21.9 ± 1.9	7.0 ± 1.7	1.2 ± 0.6	8.0 ± 4.1
Karyotype VI	8	21.7 ± 5.7	22.4 ± 4.7	18.4 ± 2.9	18.1 ± 1.7	15.5 ± 4.1	3.9 ± 1.6
Anemic							
Karyotype III	4	47.3 ± 5.1	15.4 ± 2.5	23.9 ± 1.9	7.5 ± 1.7	0.8 ± 0.5	5.2 ± 2.4
Squirrel monkey	3	28.2 ± 6.4	13.9 ± 1.6	19.6 ± 4.9	14.2 ± 4.9	10.8 ± 2.7	13.2 ± 4.4
Marmosets	3	31.0 ± 9.3	13.4 ± 0.7	20.1 ± 6.9	15.4 ± 7.3	11.8 ± 7.0	8.5 ± 2.4
Human[c]	8	24.5 ± 1.1	19.0 ± 1.2	16.4 ± 1.1	11.2 ± 1.3	15.1 ± 1.1	13.8 ± 1.3

[a] $C_{16:0}$ = palmitic acid, $C_{18:0}$ = stearic acid, $C_{18:1}$ = oleic acid, $C_{18:2}$ = linoleic acid, $C_{20:4}$ = arachidonic acid.
[b] Mean ± SD.
[c] Ref. (24).

mately 30% in normal infants to less than 20% in those experiencing hemolytic anemia. However, even though owl monkeys as a species had PE percentages less than 20%, the PUFA/SFA ratio in the resistant karyotypes was normal suggesting that peroxidation was not the primary cause of the depressed PE. Furthermore, vitamin E deficiency per se was apparently not present, since anemic owl monkeys have serum vitamin E levels (6.2 ± 2.5 μg/ml) (1) which are within the normal range (5–15 μg/ml) (26).

Another possibility is suggested by evidence from crosslinking experiments which indicate that PE and PS are incorporated in RBC membranes to a large extent as lipoproteins (27). These acidic phosphatides are thought to interact with the RBC cytoskeletal protein, spectrin, on the interior of the membrane to provide structural stability (28). Thus, a low concentration of PE and its biosynthetic precursor PS, for whatever reason, might reduce the stabilizing effects of spectrin and contribute to an unstable RBC membrane.

The striking elevation in the PC/PE ratio in the RBC membrane of owl monkeys is reminiscent of hereditary stomatocytosis and lecithin:cholesterol acyl transferase deficiency in humans. Both of these human disorders are associated with hemolytic anemia and both exhibit an absolute increase in PC levels without change in PE (19). In the owl monkey, however, the relative increase in PC was due to a decrease in the mass of PE (data not included). A similar decrease in PE in human RBCs results from a block in regeneration of PE via transfer of fatty acids to PE (29). Paysant (30) has indicated that phospholipases preferentially attack acidic phosphatides, such as PE and PS, generating the respective lysophospholipid. A compensatory regeneration pathway in the RBC membrane transfers fatty acids from PC to lyso-PE, regenerating PE, accounting for up to 30% of the PE fatty acids. This defect has been associated with hemolytic anemia in humans and is characterized by an increased PC/PE ratio. In the human condition, the PC/PE ratio increased from 1.05 to 1.77 (28). The PC/PE ratio in cebus monkeys and marmosets was 0.93 and 1.29 in squirrel and rhesus monkeys. In the owl monkey the ratio was 2.36, underscoring the magnitude of the PE decrease.

The altered RBC phospholipid profile in owl monkeys would be expected to weaken membrane integrity, but was apparently not the ultimate cause of the anemia, since the karyotype VI monkeys had the same phospholipid profile as the susceptible and anemic monkeys.

Membrane cholesterol. Cholesterol is thought to intercalate between the fatty acids of membrane phospholipids, especially unsaturated fatty acids, to make the membrane more rigid (19). Increasing the cholesterol to phospholipid ratio induces changes in surface tension and cell shape (31), reducing membrane fluidity and rendering the cell more vulnerable to hemolysis.

Although the RBCs from anemia-resistant owl monkeys (karyotype VI) had elevated cholesterol and phospholipid levels compared to humans, the FC/PL and PUFA/SFA ratios were comparable. On the other hand, the principal difference between susceptible and anemic monkeys was the tendency for increased membrane cholesterol content which led to higher FC/PL and lower PUFA/SFA ratios in RBCs of the latter. It is not apparent why the FC content of RBCs was increased in susceptible and anemic owl monkeys or in squirrel monkeys and marmosets, but the high FC/PL ratio may contribute to hemolysis. Increased membrane cholesterol is associated with hemolytic anemia in guinea pigs (32) and in humans (spur cell anemia) (33). Again, however, conflicting evidence is proffered by the high FC/PL ratio observed in RBC membranes of squirrel monkeys and marmosets in which the phospholipid profile and PUFA/SFA ratio were normal. Since these species are not afflicted with hemolytic anemia, the increased FC/PL ratio would not appear to be the cause of the anemia in owl monkeys.

Membrane fatty acids. The low PUFA/SFA ratio of susceptible and anemic owl monkeys might be attributed in part to the

phospholipid composition of the RBCs, except for the fact that resistant owl monkeys also had the altered phospholipid profile and a normal PUFA/SFA ratio. The high percentage of palmitic acid in susceptible owl monkeys was related to the high concentration of PC and Sph found in their RBCs. Palmitic acid accounted for up to one-third of the fatty acids of PC and Sph (data not included). Human RBCs also contain large quantities of palmitic acid in PC and Sph (11). In the susceptible owl monkey, low levels of arachidonic acid were found in PE (5%) and PC (2%) compared to values of 20 and 25%, respectively, in these phospholipids from human RBCs (34). Since there were no significant differences in the relative concentrations of phospholipids between karyotype VI monkeys and the other karyotypes or between the FC/PL ratio in anemic owl monkeys and healthy squirrel monkeys or marmosets, fatty acid profiles between them is considered a key to the anemia and is an undefined difference in fatty acid metabolism or destruction.

The low level of PUFA in the susceptible owl monkeys suggests that significant reduction in these membrane fatty acids occurs before the anemia is manifest. In this context it is noteworthy that essential fatty acid deficiency is associated with hemolytic anemia in man (4). If fatty acid peroxidation accounts for the decreased PE and PS levels, it would appear that karyotype VI monkeys can either resist PUFA destruction or replace membrane phospholipid PUFA more efficiently than the other karyotypes. In the anemia of human vitamin E deficiency, surviving cells incorporate labeled fatty acids into PE approximately 40% more efficiently than vulnerable hemolyzing cells (14). A deficiency of this acylating enzyme in susceptible karyotypes could induce the anemia.

Lipid abnormalities encountered in RBCs from several human hemolytic anemias were observed in RBCs of susceptible and anemic owl monkeys. An increased FC/PL ratio is exhibited in LCAT deficiency (20), spur cell anemia (33), and cholesterol-induced anemia in the guinea pig (32). Low red cell PUFA levels and a decrease in the inner membrane phospholipids are associated with vitamin E deficiency anemia (22–25), and a high PC/PE ratio is present in RBCs in LCAT deficiency (20), vitamin E deficiency (22–25), and hereditary stomatocytosis (19). Elucidation of the etiologic factors in the pathogenesis of the hemolytic anemia in owl monkeys should provide meaningful information on mechanisms operative in hemolytic anemias in man.

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