PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 153, 505-510 (1976)

Spiculated Erythrocytes of Cholesterol-Fed Guinea Pigs: Changes of Morphology, Composition, and Osmotic Fragility[1] (39579)

CAROLYN ASWAD AND ROSEMARIE OSTWALD

Department of Nutritional Sciences, University of California, Berkeley, California 94720

Dietary cholesterol induces a hemolytic anemia in guinea pigs (GP) preceded for many weeks by fatty infiltration of the liver, by increases in liver, plasma, and erythrocyte (RBC) cholesterol contents, and by changes in RBC morphology (1, 2). The cause for the morphological abnormalities of cholesterol-loaded RBC is not well understood, but changes in cell-shape have been reported to accompany increased membrane cholesterol both in human pathologies (3-5) and in *in vitro* systems (6, 7). A causal relationship would be strengthened if the time course of shape change and alteration of cholesterol content could be shown to be similar. Maintenance of normal red cell shape has been shown to require normal levels of ATP and ATPase activity (8, 9) and therefore of proper functioning of energy-producing reactions. Changes in membrane composition may be associated with alterations of enzyme activities. This study was designed to compare sequential changes in the morphology with the cholesterol content of guinea pig RBC in response to dietary cholesterol. Concentration of ATP and the activities of three key enzymes in the glycolytic sequence and of ATPases were monitored simultaneously.

The mechanism for the development of the hemolytic anemia in cholesterol-fed (CHOL) GP has not been established. Changes in fragility of cholesterol-loaded RBC might be one possible mechanism. Osmotic fragility has been shown to be altered in patients with obstructive jaundice and alcoholic liver disease associated with an increase of RBC cholesterol (4, 10). We therefore measured changes in the osmotic fragility of guinea pig RBC in response to dietary cholesterol.

[1] This work was supported in part by USPHS, NIH Grant No. 08480, and was presented in part at the 58th Annual Meeting of FASEB, Atlantic City, April 1974.

Materials and methods. Male guinea pigs (200 g) were fed *ad libitum* Purina guinea pig chow supplemented with 5% cottonseed oil for 4 weeks prior to the addition of 1% cholesterol to the diet of the experimental group (11). Blood was collected either directly from the heart or from the ear vein using disodium ethylenediaminetetraacetic acid (EDTA) or heparin as anticoagulant, respectively.

Morphology of the RBC was assessed in whole blood. One drop of blood was diluted into 1.0 ml of Hayem's solution (9 m*M* $HgCl_2$, 75 m*M* Na_2SO_4, 85 m*M* NaCl). Slides of fixed cells were photographed using phase contrast optics and RBCs were classified into categories as summarized in Fig. 1.

Cholesterol of plasma and RBC, washed twice with isotonic saline, was extracted, precipitated as the digitonide, and assayed using the ferric chloride colorimetric method (12).

Osmotic fragility of red cells was determined in freshly drawn whole blood. Blood was added to tubes containing varying concentrations of NaCl (0-0.75%, w/v). The absorbance of the supernatant solutions was read at 540 nm after 60 min.

ATP content of whole blood, and the activities of hexokinase (Hex), phosphofructokinase (PFK), pyruvate kinase (PK), and of Na^+-, K^+-, and Mg^{2+}-stimulated ATPases were measured by published methods (13, 14).

Results. Morphology and cholesterol content. Cholesterol content in RBC of CHOL GP increased sharply within 1 to 3 days. Plasma cholesterol also increased during this period. After the first week, only small increases in both RBC and plasma cholesterol content were observed until the onset of the anemia several weeks later (Table I). In control guinea pigs (CONT GP), cholesterol content of RBC and plasma remained

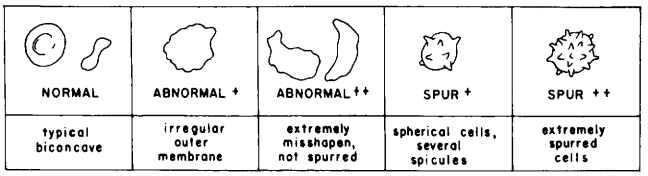

FIG. 1. Morphological classifications of red cells. Morphology of erythrocytes was assessed in whole blood obtained from the ear vein. One drop of blood was diluted in Hayem's solution, photographed using phase contrast optics, and counted by class.

TABLE I. SEQUENTIAL CHANGES OF PLASMA AND ERYTHROCYTE CHOLESTEROL CONTENT AND OF ERYTHROCYTE MORPHOLOGY DURING 15 WEEKS OF CHOLESTEROL FEEDING.

		Days								
		−1	+1	+3	+5	+7	+13	+21	+35	+85
Cholesterol[a] (mg/100 ml)										
RBC										
Expt. 1[b]	CONT	121(7)[c]	141(3)^{y}	114(3)^{y}	123(3)^{x}	141(2)		124(5)	126(5)	135(3)
	CHOL	123(9)	159(4)^{y}	^{x}145(5)^{y}	^{x}185(4)^{y}	^{x}181(5)		^{x}193(8)	^{x}201(8)	^{x}283(6)
Expt. 2	CONT	159(4)	164(4)		165(4)		149(3)		119(4)	146(4)
	CHOL	160(4)	^{x}191(4)^{y}		^{x}214(4)^{y}		173(2)^{y}		^{x}240(4)^{y}	
Plasma										
Expt. 1	CONT	37(7)	48(3)	47(3)	47(3)	64(2)		60(5)	50(4)	52(3)
	CHOL	^{x}58(9)	^{x}67(4)	^{x}75(5)^{y}	^{x}189(4)^{y}	^{x}214(5)		^{x}191(8)	^{x}193(8)	^{x}216(6)
Expt. 2	CONT	47(4)	47(4)		57(4)					
	CHOL	59(4)	^{x}101(4)^{y}		–					
Morphology[d]										
Expt. 1	CHOL									
Abnormal (%)		11(5)	30(5)	17(5)	25(5)	36(5)		38(5)	24(5)	29(6)
Spur (%)		0	0	1	3	5		48	66	56
Expt. 2	CHOL									
Abnormal (%)		14(2)	30(4)		27(4)		74(4)		39(4)	
Spur (%)		0	0		0		9		60	

[a] Cholesterol content of erythrocytes (RBC) and plasma was determined at intervals after initiation of the cholesterol-containing diet in control (CONT) and cholesterol-fed (CHOL) guinea pigs. In experiment 1, blood was sampled by closed heart puncture with EDTA as anticoagulant; in experiment 2, it was sampled by ear vein with heparin as anticoagulant.

[b] Two different subgroups were bled on Days 1 and 5 or 3 and 7 in order to avoid undue stress and blood loss.

[c] Mean (number of animals); x, CHOL is different from CONT at $p < 0.02$; y, mean is different from preceding time point at $p < 0.02$.

[d] Morphology of Hayem's-fixed red cells from whole blood of CHOL GP (see Materials and Methods). For classification, see Fig. 1. Abnormal, Ab^{+} plus Ab^{++}; Spur, $Spur^{+}$ plus $Spur^{++}$. Mean (number of animals) of fractions of cells in each class. Total number of cells on each slide was 30–100 cells. The range of values for cells from CONT GP were as follows: % normal, 70–90; % Ab^{+}, 6–20; % Ab^{++}, 0.8; % $Spur^{+}$, 0.5; % $Spur^{++}$, 0.

unchanged during the experimental period with some fluctuations in that of the RBC.

Increased RBC cholesterol content was associated with changes in morphology (Fig. 2). After 1 day of dietary cholesterol, 30% of the cells were characterized by wavy perimeters (Ab^{+} and Ab^{++}; Fig. 1; Table I). Significant spurring occurred first between Weeks 1 and 3 despite only minor additional increases of the membrane cholesterol content. A maximum of 65% echinocytes was observed at Week 5.

Osmotic fragility and cholesterol content. Osmotic fragility of RBC decreased simultaneously with increases of membrane cholesterol content. After 1 day of feeding cholesterol, osmotic fragility of CHOL RBC was significantly less than that of CONT RBC ($P < 0.01$) and decreased further to Day 5 (Fig. 3).

Energy metabolism and cholesterol content. ATP content of RBC and activities of ATPases, PK, Hex, and PFK, were found to be correlated with the percentage of reticulocytes ($r = 0.82$–0.95), and were not correlated with either the cholesterol content nor the morphological abnormalities.

Hemolysis, morphology, and cholesterol content. Red cell counts began to fall and the percentage of reticulocytes started to rise from 5–9 weeks after the start of cholesterol supplementation. The overt anemia

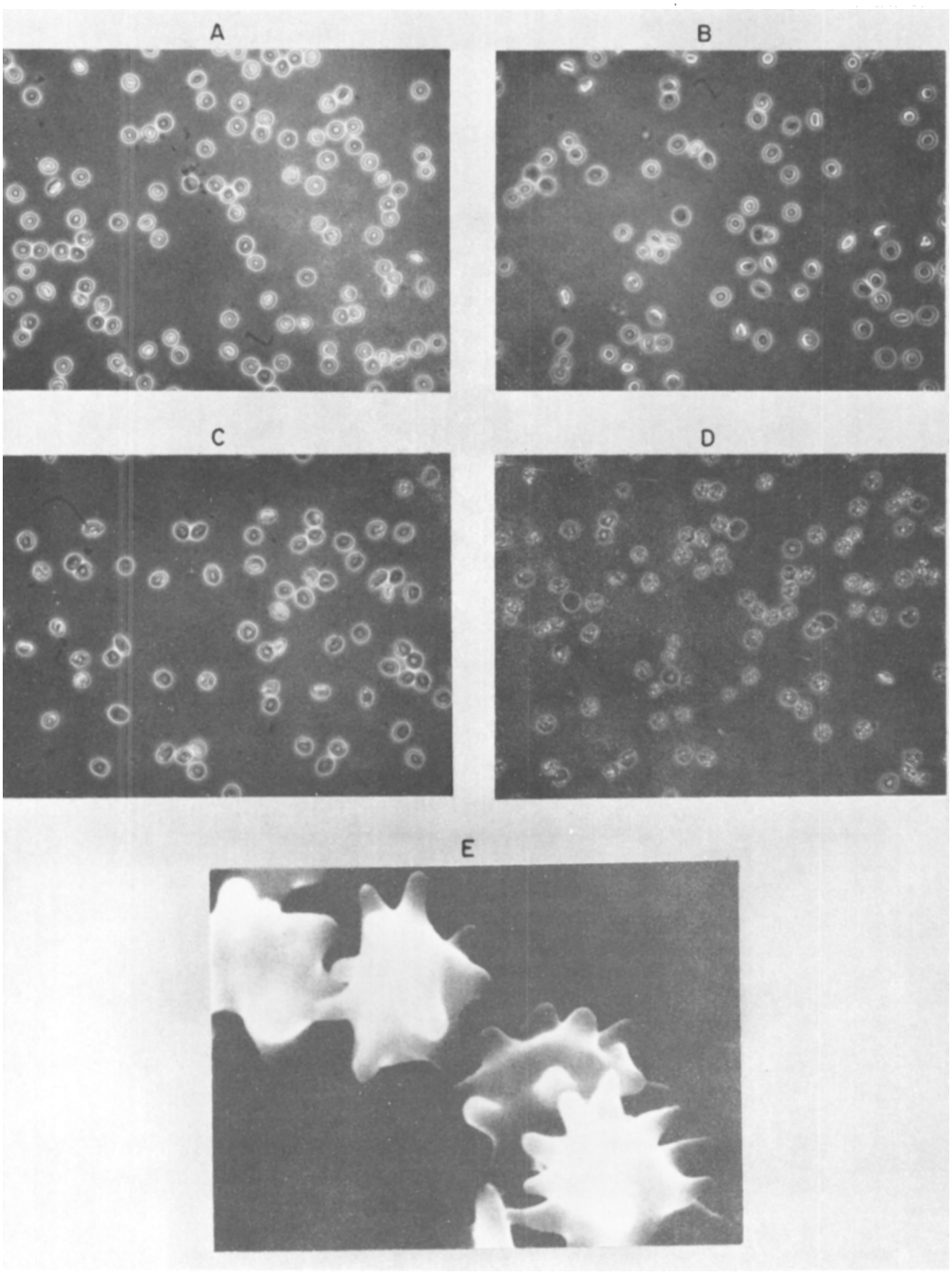

FIG. 2. Electronmicrographs of guinea pig red cells. (A–D) Red cells of guinea pigs fed cholesterol for −1, +5, +7, and +21 days, respectively. Whole blood was fixed in Hayem's solution. Polaroid photographs using phase contrast optics (× 280). (E) Red cells of a cholesterol-fed, anemic guinea pig. Scanning electronmicrographs (× 7000).

(RBC count less than 3.5 × 10^6 cell/mm^3) generally occurred 4–6 weeks later (Table II). The time varied between animals and between different groups of these outbred guinea pigs. The number of reticulocytes in CONT red cell populations was consistently less than 1% and the RBC counts averaged between 4.9 and 5.8 × 10^6 cells/mm^3. This time course for the development of the hemolytic anemia is in contrast to the very early changes is red cell cholesterol content, osmotic fragility, and morphological abnormalities.

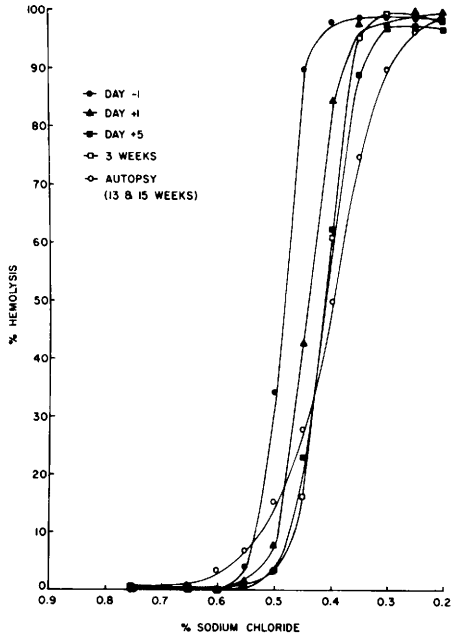

FIG. 3. Osmotic fragility of RBC from guinea pigs fed 1% cholesterol for different times as indicated (see Materials and Methods). Points are means of four to five animals. The concentration of NaCl for 50% hemolysis was 0.50, 0.44, 0.44, 0.41, 0.41, 0.41, 0.40, and 0.40% for Days −1, +1, +3, +5, +7, +21, and Weeks 5 and 13, respectively. The corresponding values for CONT cells were 0.50, 0.49, 0.47, 0.49, 0.48, 0.47, 0.47, and 0.47%. Differences between CONT and CHOL cells were statistically different at $P < 0.01$ from Day +1 on.

Discussion. We have previously reported a doubling of the cholesterol content of erythrocytes in cholesterol-fed anemic guinea pigs (1, 2). In the present study, red cell cholesterol was shown to increase within 1–3 days after supplementation of the diet with 1% cholesterol simultaneously with the increase of plasma cholesterol content. The rate of cholesterol accumulation by the red cells remained parallel to the increases in plasma cholesterol throughout the experimental period.

Morphological alterations and elevations of membrane cholesterol similar to those observed in CHOL GP can be produced *in vitro* when RBC from CONT GP are incubated in plasma from CHOL GP (6) and when human RBC are incubated in plasma from patients with spur cell anemia or with cholesterol-rich lipid dispersions (7). Our evidence, as well as that of others, indicates that the factor or factors in these plasma responsible for the net transfer of CHOL to the RBC and for the subsequent morphological abnormalities are abnormal lipoproteins with a high CHOL/PL ratio (4–7).

The appearance of Ab^+ and Ab^{++} forms occurred simultaneously with the initial increase of red cell cholesterol. $Spur^+$ and $spur^{++}$ forms appeared about 2 weeks later, in the absence of further substantial increases of red cell cholesterol content. These abnormal RBC resemble the crenated disk-crenated spheres and the discocyte–echinocyte transformations described earlier (15, 16). If we assume that the

TABLE II. RED CELL COUNTS AND PERCENTAGES OF RETICULOCYTES IN CHOLESTEROL-FED GUINEA PIGS.[a]

	Expt. 1					Expt. 2			
Weeks	1	3	5	9	12	3	5	7	9
RBC per mm^3 × 10^6	5.2	5.8	5.4	4.8	3.5	4.9	4.7	3.6	3.5
Reticulocytes %			0.8	4.1	10.0	1.2	1.5	15.7	18.5
	Expt. 3					Expt. 4			
Weeks		9	11	13		5	11	14	
RBC per mm^3 × 10^6		4.8	4.6	3.5		5.9	3.6	3.6	
Reticulocyte		4.1	6.6	9.3			16.7	22.6	

[a] Red cell (RBC) and reticulocyte counts in guinea pigs when fed a 1% cholesterol-containing diet for varying periods of time in our replicate experiments are given; mean of four to six animals. Red cell counts in control guinea pigs averages 4.9–5.8 × 10^6 cells per mm^3 whole blood. Reticulocytes were less than 1%.

events *in vivo* are analogous to those observed *in vitro*, we could conclude that spicule formation in the cholesterol-fed GP is associated with an increased cholesterol content of the red cell membrane and is the end result of a series of sequential shape transformations. Proof of this hypothesis has to await the demonstration that the forms Ab^{+}, Ab^{++}, $spur^{+}$, and $spur^{++}$ are in fact transformations of the same cell.

The morphological abnormalities of the cholesterol-loaded GP RBC do not seem to be related to changes in the activities of key enzymes necessary for the energy metabolism of the cell nor to a depletion of ATP (8, 9). The changes which we did observe occurred long after the appearance of the echinocytes and were correlated with the reticulocytosis. Reticulocytes and young red cells have been reported to have higher concentrations of ATP (17) and increased activities of glycolytic enzymes (18) than do mature RBC.

An inverse association of osmotic fragility and cholesterol content has been reported by Cooper in patients with alcoholic liver disease and has been shown to be related to an increase of surface area (7, 10). Calculation of surface area of the CHOL RBC from the critical hemolytic volume (19) and on the basis of an unchanged MCV of CHOL RBC (2) indicate an increase of 4–8%, correlating with the percentage increases of membrane cholesterol ($r = 0.89$).

Reduced osmotic fragility, possibly related to the increased local viscosity of cholesterol-loaded RBC (20) as well as to the increased surface area, might lead to decreased RBC deformability, thereby increasing membrane fragmentation and splenic sequestration of red cells. Spiculation of RBC might also be expected to decrease the ease of red cell filtration through the reticuloendothelial system. However, it seems unlikely that either the reduced osmotic fragility or spiculation of the cholesterol-loaded RBC contributes to the eventual appearance of the hemolytic anemia. Although a major proportion of GP RBC was severely spiculated after 3 weeks of feeding cholesterol, the overt hemolytic anemia did not occur until an additional period of 6 to 9 weeks of cholesterol supplementation. We have shown previously that the earliest signs of an anemia as indicated by bone marrow response, hematological parameters, and decrease of mean survival time of RBC appear at about Week 7 (2). Furthermore, splenectomy either before, during, or after initiation of the cholesterol-containing diet has no effect on the development of the anemia (21; W. Yamanaka and R. Ostwald, unpublished observations). Some other mechanism must be involved in the eventual development of the hemolytic anemia in cholesterol-fed guinea pigs.

Summary. We have studied the sequential changes of cholesterol content and morphology in RBC of cholesterol-fed GP and the effects of these changes on their energy metabolism and osmotic fragility. The results suggest that (i) spurring of GP RBC is not accompanied by obvious alterations in their energy metabolism and may be the end result of sequential shape transformations associated with cholesterol-loading, and (ii) neither the morphological abnormalities nor the decreased osmotic fragility of cholesterol-loaded RBC is directly related to the development of the hemolytic anemia.

Our thanks go to Dr. T. Hayes, Donner Laboratory, University of California, Berkeley, for the scanning electronmicrograph and for permission to publish it. The replicate experiment (Table I, Expt. 2) was carried out by Jacqueline Fields.

1. Ostwald, R., and Shannon, A., Biochem. J. **91,** 146 (1969).
2. Ostwald, R., Yamanaka, W., Light, M., and Kroes, J., Atherosclerosis **23,** (1976) in press.
3. McBride, J. A., and Jacobs, H. S., Brit. J. Haematol. **18,** 383 (1970).
4. Cooper, R. A., J. Clin. Invest. **48,** 1820 (1969).
5. Norum, K. R., and Gjone, E., Scand. J. Clin. Lab. Invest. **22,** 94 (1968).
6. Sardet, C., Hansma, H., and Ostwald, R., J. Lipid Res. **13,** 705 (1972).
7. Cooper, R. A., Arner, E. C., Wiley, J. S., and Shattil, S. J., J. Clin. Invest. **55,** 115 (1975).
8. Nakao, M., Nakao, T., Yamazoe, S., and Yoshikawa, H., J. Biochem. **49,** 487 (1961).
9. Szasz, I., Arky, I., and Gardos, G., Folia Haemat. **89,** 501 (1968).
10. Cooper, R. A., and Jandl, J. H., J. Clin. Invest. **47,** 809 (1968).
11. Matin, C., and Ostwald, R., J. Nutr. **105,** 525 (1975).
12. Zlatkis, A., Zak, B., and Boyle, A., J. Lab. Clin.

Med. **41,** 486 (1953).
13. Beutler, E., "Red Cell Metabolism." Grune and Stratton, New York and London (1971).
14. Brewer, G. J., Eaton, J. W., Beck, C. C., Feitler, L., and Shreffler, D. C., J. Lab. Clin. Med. **71,** 744 (1968).
15. Deuticke, R., Biochem. Biophys. Acta **163,** 494 (1968).
16. Brecher, G., and Bessis, M., Blood **40,** 333 (1972).
17. Shojania, A. M., Israels, L. G., and Zipursky, A., J. Lab. Clin. Med. **71,** 41 (1968).
18. Brok, F., Ramot, B., Zwang, E., and Dannon D., Israel J. Med. Sci. **2,** 291 (1966).
19. Castle, W. B., and Daland, G. A., Amer. J. Physiol. **120,** 371 (1937).
20. Kroes, J., Ostwald, R., and Keith, A., Biochem Biophys. Acta **274,** 71 (1972).
21. Kennedy, B., and Okey, R., Amer. J. Physiol **149,** 1 (1947).

Received February 23, 1976. P.S.E.B.M. 1976, Vol 153.