Red cell cholesterol enrichment and spur cell anemia in dogs fed a cholesterol-enriched, atherogenic diet

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Abstract A diet supplemented with cholesterol and coconut oil is atherogenic in dogs. The purpose of the present study was to examine the effects of this diet on red cells in pure-bred beagles and greyhounds. Within 3 days after the initiation of this diet red cell cholesterol/phospholipid increased and membrane fluidity decreased, with maximum changes attained by 12 weeks. Serum lipoprotein cholesterol/phospholipid also increased, and serum from cholesterol-fed dogs transferrred cholesterol to normal red cells. Significant abnormalities of liver function developed in all cholesterol-fed dogs. Hematocrit declined beginning at 6 weeks, with a parallel increase in osmotic fragility. Reticulocytes were elevated in beagles but normal in greyhounds. Red cell morphology resembled acanthocytes or spur cells. All red cell parameters returned to normal within 4 weeks after stopping the diet. These studies demonstrate that a cholesterol-enriched, atherogenic diet causes profound and reversible changes in the lipid composition, membrane fluidity, and morphology of red cells in dogs. — Cooper, R. A., M. H. Leslie, D. Knight, and D. K. Detweiler. Red cell cholesterol enrichment and spur cell anemia in dogs fed a cholesterol-enriched, atherogenic diet. J. Lipid Res. 1980. 21: 1082–1089.

Supplementary key words red cell membrane, cholesterol/phospholipid, membrane fluidity, atherogenesis, cholesterol feeding

Cholesterol enrichment of red cell membranes associated with hemolytic anemia occurs in several rodent species fed diets enriched with cholesterol. This was first described in guinea pigs by Okey and Greaves (1), and it subsequently has been investigated in this species by Ostwald and her associates (2–4). Similar observations have been made in rabbits (5, 6). A characteristic red cell morphologic defect, referred to as acanthocytes or spur cells, accompanies the red cell lipid abnormality. This red cell defect in both guinea pigs and rabbits is similar to the syndrome of spur cell anemia which occurs spontaneously in some patients with severe hepatic dysfunction (7). Shull and his coworkers (8) have described an identical syndrome occurring spontaneously in a mixed-breed dog with severe hepatic disease.

Administration of a cholesterol-enriched diet to dogs has been reported to induce hypercholesterolemia (9) and atherosclerosis (10, 11). However, Butkus and his coworkers (12) concluded that this diet did not alter red cell cholesterol, nor did it induce anemia. It was therefore a surprise when, in the course of administering this diet to pure-bred beagles and greyhounds, a beagle developed profound spur cell hemolytic anemia. This prompted us to reexamine the effect of this cholesterol-enriched, atherogenic diet on red cells in dogs.

METHODS

The animals used in this study were all adult beagles and racing greyhounds approximately 2 to 4 years of age at the time of the study. The control dogs were fed a pelleted commercial diet (Wayne Dog Food, Allied Mills, Inc., Chicago, IL). The treated dogs received a pelleted modification of the Malmros-Sternby diet (13). This is a semi-synthetic diet containing 16% coconut oil and 5% cholesterol (test diet TD 73427 from Tekland Mills; Madison, WI) which are the essential constituents required to produce atherosclerosis in euthyroid dogs, as shown by Malmros and Sternby (13). The present version of the diet was evolved from studies employing this basic diet in which certain side effects such as silica

Abbreviations: C/P, cholesterol/phospholipid mole ratio; r, fluorescence anisotropy; DPH, 1,6-diphenyl,1,3,5-hexatriene; P, fluorescence polarization.

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urolithiasis have been eliminated (10, 11, 14). The significant features of this diet are a deficiency in essential fatty acids and the high concentration of saturated fat. The dogs were given free access to their food and intake was determined by weighing the amount remaining each 24 hr. All animals were given periodic clinical examinations including blood pressure determination, ECG, blood, and urinalysis, etc. They were housed in a large kennel room in individual cages with outdoor runs (Federated Medical Resources, Honeybrook, PA).

Periodic blood samples were obtained by venipuncture, and anticoagulated with heparin. Red cell morphology was assessed on Wright’s stained smears. Osmotic fragility was carried out as previously reported (15). Red cells were freed of plasma and buffy coat by washing thrice with normal saline. Quadruplicate aliquots of red cells were extracted with 80 volumes of isopropanol and chloroform. Cholesterol was determined by the method of Zlatkis, Zak, and Boyle (17) after washing the extracts free of non-lipid phosphorus. Lipid phosphorus was measured by the method of Bartlett (18). Individual phospholipid classes were separated on thin-layer chromatography according to Skipski, Peterson, and Barclay (19).

For measurements of fluorescence anisotropy, red cell ghosts were isolated by the method of Dodge, Mitchell, and Hanahan (20). The fluorescent probe, 1,6-diphenyl-1,3,5-hexatriene (DPH) (Aldrich Chemical Co., Milwaukee, WI), was used to label red cell ghosts. Measurements of fluorescence polarization and fluorescence intensity were performed with an Elscint MV-1 microviscosimeter (Elscint Corp., Hackensack, NJ) equipped with a thermoregulated sample chamber (21, 22). Temperature was measured with an electronic thermistor (Cole-Palmer Instrument Co., Chicago, IL).

DPH was kept as a stock solution in tetrahydrofuran at a concentration of $2 \times 10^{-3}$ M. Immediately prior to use, it was diluted 1:2000 in 0.155 M NaCl with vigorous mixing. One volume of the dilute DPH dispersion was added to one volume of red cell ghosts suspended in 0.155 M NaCl at a ghost concentration of $1 \times 10^8$ per ml, and the mixture was incubated at 37°C for 30 min.

The microviscosimeter simultaneously analyzes $I_\parallel$ and $I_\perp$ and calculates and displays the degree of fluorescence polarization ($P$):

$$P = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp} \quad (1)$$

Fluorescence anisotropy ($r$) is calculated according to the relationship:

$$r = \frac{I_\parallel - 1}{I_\parallel + 2I_\perp} \quad (2)$$

Plasma lipids were extracted with acetone-ethanol 1:1 at 50°C for measurement of total (17) and unesterified cholesterol (23) and lipid phosphorus (18). For both red cells and plasma, phospholipid values were taken to equal lipid phosphorus times 25. Lipoprotein density classes were separated from plasma by ultracentrifugation after density adjustment with KBr (24).

To test the effect of cholesterol-enriched plasma on normal red cells, red cells from a normal greyhound were washed three times with Hank’s balanced salt solution, resuspended in Hank’s to a hematocrit of 10%, and incubated with an equal volume of plasma containing penicillin, 1,000 U/ml. Incubations were performed in stoppered, sterile $16 \times 150$ mm test tubes in a 37°C atmosphere with shaking at 130 oscillations/min for 20 hr. Thereafter, red cells were washed free of plasma and extracted for measurements of lipids, as described above. Data in the text are expressed as mean ± SE.

RESULTS

Severe anemia occurring in a cholesterol-fed beagle called our attention to this disorder in dogs. This animal was studied longitudinally. When initially studied, this animal had been receiving a cholesterol-enriched diet for 16 weeks. His initial hemoglobin was 7.9 g/dl and reticulocytes were 2.4%. The red cell morphology was characteristic of spur cell anemia as it occurs in man and dogs and as it develops when rabbits and guinea pigs are fed cholesterol (Fig. 1). The initial red cell cholesterol was 23.9 µg/10⁸ cells (normal = 13.6 ± 0.3 µg/10⁸ cells). The cholesterol/
Fig. 2. Fluidity of red cell membranes as influenced by cholesterol feeding. Data reported as fluorescence anisotropy of 1,6-diphenyl-1,3,5-hexatriene (DPH) at 37°C. Increased fluorescence anisotropy reflects decreased fluidity.

Phospholipid mole ratio of these red cells was increased from the normal values of 1.04 ± 0.02 to 1.53. Fluorescence anisotropy of DPH was increased from the normal values of 0.186 ± 0.001 to 0.216. These initial observations suggested that cholesterol feeding led to an enrichment of red cell membranes with cholesterol, leading to a decreased membrane fluidity as evidenced by an increase in the fluorescence anisotropy of DPH. This was associated with spurious cell hemolytic anemia. The animal was immediately switched to a control diet.

Three months after instituting a normal diet, all of these determinations had returned to normal. At that time the cholesterol-enriched diet was begun again, and serial measurements were carried out. Red cell C/P rapidly increased to 1.14 on day 3 with a corresponding increase in fluorescence anisotropy (Fig. 2) and changes in red cell morphology (Fig. 1). Thereafter, both values continued to increase to maximum levels for C/P of 1.38 and maximum values for fluorescence anisotropy of 2.15 at 12 weeks. Red cell spiculation continued with no striking change from earlier observation points. With continuation of the diet, values for C/P remained in the range between 1.33 and 1.38, and fluorescence anisotropy remained elevated. An analysis of the fluorescence anisotropy of DPH over a range of temperatures was carried out at 20 weeks (Fig. 3). Values were increased in the cholesterol-fed animal throughout the temperature range studied, and no phase transition was observed. At 24 weeks, a normal diet was re instituted. Membrane fluidity rapidly increased with a full return of fluorescence anisotropy to normal within 4 weeks. Membrane C/P decreased to 1.13 at 2 weeks after re instituted a normal diet.

Both the hematocrit and the red cell osmotic fragility remained unchanged during the first 6 weeks of dietary cholesterol administration (Fig. 4). Thereafter, osmotic fragility progressively increased, associated temporally with a falling hematocrit. Changes in osmotic fragility were maximal by the 20th week. Upon cessation of the cholesterol-enriched diet, red cell osmotic fragility rapidly returned to normal, and the hematocrit rose to normal levels. Thus, in this beagle, there was the development of cholesterol-rich, distorted red cells displaying an increased membrane C/P, decreased membrane fluidity, and increased osmotic fragility in association with anemia, and all of these characteristics reverted to normal upon reintroduction of a normal diet.

To determine if the observations in this beagle were unique to this one animal or if this phenomenon was a general manifestation of cholesterol administration to dogs, four additional beagles and nine racing greyhounds were fed the identical diet for 6 months. The data are summarized in Table 1. Hematocrit levels fell in both species at 6 months, but the fall was more marked in beagles than in greyhounds. A small, but significant, reticulocytosis occurred in beagles.
whereas there was no significant change in the reticulocyte count in greyhounds. Red cell morphology similar to that depicted in Fig. 1 occurred in all dogs fed the cholesterol-enriched diet.

There was a consistent increase in red cell cholesterol with only slight increases in the total amount of red cell phospholipid in both species. This resulted in an increase in the C/P of approximately 25%. There were no significant changes in the relative quantities of the various phospholipids. Values for fluorescence anisotropy increased in parallel with the changes in C/P.

Plasma lipids were also markedly affected by the administration of a cholesterol-enriched diet (Table 2). Unesterified cholesterol levels rose markedly, as did cholesterol esters, but phospholipid levels increased to a lesser degree. The lipid composition of individual lipoprotein classes was analyzed in pooled plasma from the nine cholesterol-fed greyhounds. Only very small amounts of lipid were found in density fractions less than 1.063 g/ml in the control animals, and these amounts did not permit accurate quantitation. Significant quantities of lipid existed in these lower density fractions in the cholesterol-fed animals. The C/P of the high density fraction increased from 0.28 in control animals to 0.41 in the cholesterol-fed animals, and lipoproteins containing approximately equimolar cholesterol and phospholipid were found in the lower density fractions.

To test whether this cholesterol-enriched plasma directly affected normal red cells, the red cells from a control greyhound were incubated with plasma pooled from cholesterol-fed greyhounds. The C/P of these red cells prior to incubation was 0.98, and it increased to 1.27 after 20 hr incubation in the presence of this cholesterol-enriched plasma.

Abnormalities of liver function were evident within one week of instituting the cholesterol-enriched diet, and they continued throughout the period of cholesterol feeding. Values at 24 weeks are shown in Table 3. These values are similar to the values obtained at 12 weeks. After one year of exposure to this cholesterol-enriched diet, all animals were killed. At that time atherosclerosis was present in all animals, but to a somewhat greater degree in greyhounds. The livers of the cholesterol-fed animals exhibited prominent Kupffer cells, fatty degeneration of hepatocytes, ductal proliferation, and focal periportal regeneration.

### TABLE 2. Lipoprotein lipids of control and cholesterol-fed greyhounds at 24 weeks

<table>
<thead>
<tr>
<th>Lipoprotein Type</th>
<th>Control (5)</th>
<th>Cholesterol-Fed (9)</th>
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<tbody>
<tr>
<td>Cholesterol, mg/dl</td>
<td>110 ± 13</td>
<td>583 ± 140</td>
</tr>
<tr>
<td>Esterified cholesterol, mg/dl</td>
<td>363 ± 32</td>
<td>790 ± 44</td>
</tr>
<tr>
<td>Phospholipids, mg/dl</td>
<td></td>
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The levels of lipoproteins of densities less than 1.063 g/ml were too low for analysis in control animals. Cholesterol feeding led to cholesterol enrichment of d = 1.063–1.21 g/ml lipoproteins and to the appearance of lower density lipoproteins with approximately equimolar cholesterol and phospholipid. Data are expressed in terms of the concentration of each constituent in a volume equal to that from which the lipoproteins were isolated.

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tion. Thus, this cholesterol-enriched diet had a profound and deleterious effect on both liver architecture and hepatic function.

**DISCUSSION**

These studies demonstrate that a cholesterol-rich, atherogenic diet in dogs simultaneously induces cholesterol-enrichment of red cell membranes and the syndrome of spur cell anemia. It has generally been felt that cholesterol enrichment of red cells in animals fed lipid-supplemented diets was unique to rodents (25). Even among rodents, only rabbits and guinea pigs had been clearly demonstrated to develop cholesterol-enriched red cells when fed diets supplemented with cholesterol. Rats have been particularly resistant to cholesterol-enriched diets, with or without corn oil supplements. In either case, little or no change occurs in the red cell membrane cholesterol content in rats (26). Mice fed a diet enriched with both cholesterol and cholic acid develop cholesterol enrichment of red cell membranes with the corresponding changes in membrane fluidity, but the morphologic abnormality seen in the present study and in previous reports in rabbits and guinea pigs does not occur in such mice (27).

Some variability appears to exist even in the canine species. In studies with mongrel dogs, Butkus and coworkers (12) found that animals fed a diet identical to that used in the present study for a period of 12 months developed an increased red cell osmotic fragility but no change in hematocrit. The beagles in the present study developed a more severe degree of anemia than the greyhounds, and among the beagles studied, one specific animal developed a degree of anemia out of proportion to the other animals. Similarly, among patients with hepatocellular disease of seemingly equivalent degrees of severity, the occurrence of spur cells and anemia is a very inconsistent finding, occurring in no more than 5–10% of such patients (28).

The finding of red cell cholesterol enrichment in the present study is at variance with the red cell lipid values reported by Butkus and coworkers previously (9). These workers interpreted their data as showing no change in red cell cholesterol or phospholipid after 12 months of feeding the same diet used in the present study to a group of mongrel dogs. However, the normal value for C/P in their study was 0.64, a distinctly low value for red cells from any mammalian species (29) and a value distinctly lower than that observed in the beagles and greyhounds in the present study. While it is possible that the differences between the present study and that previously reported by Butkus and coworkers (9) represent variation due to animal species, it is more likely that they represent methodologic differences.

The diet used in this study was not only enriched with cholesterol but was also enriched with coconut oil and was deficient in essential fatty acids. Previous studies with this diet have demonstrated that only minor changes occur in red cell fatty acids after 12 months of continued exposure, a decrease in 18:O and 20:4 fatty acids occurs associated with a reciprocal increase in 18:1 and 20:3. Although we did not carry out fatty acid measurements in the group reported herein, it is likely that within the time frame of these experiments, little change occurred in red cell fatty acids.

The changes in fluidity observed in the present study are similar in magnitude to those observed under other conditions of red cell cholesterol enrichment. These have included red cells obtained from patients with spur cell anemia, red cells incubated in the plasma of such patients, and red cells incubated in a medium supplemented with liposomes having a C/P ration of greater than 1.0 (21, 30). These changes in fluidity are also similar to those observed in mice fed a diet supplemented with cholesterol and cholic acid (27). This confirms the general phenomenon that cholesterol decreases the mobility of lipids in the hydrophobic core of the membrane thus creat-
ing a more viscous microenvironment in that portion of the bilayer probed by DPH. It is not clear whether it is the change in membrane fluidity per se or some other simultaneous change in the membrane associated with cholesterol enrichment which is responsible for the abnormal morphology of red cells in this disorder.

The increased C/P of serum lipoproteins observed in the present study is analogous to observations previously made in guinea pigs (31) and rabbits (32). In guinea pigs, the predominant change appears to occur in the lipoprotein of density 1.006–1.063 g/ml, whereas in rabbits the major change appears to occur in a species of lipoproteins of density >1.070 g/ml. The greyhounds studied herein underwent an increase in the cholesterol content and the C/P of lipoproteins in the density class 1.063–1.21 g/ml. In addition, they acquired substantial quantities of lipoproteins which were isolated in lower density fractions and which had approximately equimolar concentrations of cholesterol and phospholipid.

The ability of plasma from cholesterol-fed greyhounds to transfer cholesterol to red cells during a period of 20 hr of incubation in vitro again points up the similarities between this phenomenon in dogs and the phenomena studied previously in cholesterol-fed guinea pigs (4) and in patients with liver disease (7). Thus, it appears that cholesterol feeding induces a primary change in lipoprotein composition that, by exchange equilibrium, imparts a chemical change to red cells leading, in turn, to a change in membrane architecture and a sensitivity to destruction in vivo.

Previous workers have described a pattern of liver injury in rabbits and guinea pigs fed a cholesterol-enriched diet (33, 34). This is characterized by centrilobular fatty deposition leading later to diffuse portal cirrhosis. Birefringent crystals, presumably cholesterol, were observed within the liver. The pattern of liver injury in the dogs in the present study was similar. It is not known how important this liver insult is to the body’s handling of ingested cholesterol so as to maintain circulating lipoproteins which have a high C/P. Since liver disease that occurs spontaneously in both man and dogs in the absence of excess dietary cholesterol leads to the development of cholesterol-enriched lipoproteins (7, 8), the role that hepatic damage in these cholesterol-fed animals plays in the sequence of events leading to cholesterol-enrichment of plasma lipoproteins and red cell membranes cannot be discounted.

In previous studies in vitro, the addition of cholesterol to red cell membranes has expanded the surface area and decreased red cell osmotic fragility (7, 35). In contrast, the osmotic fragility of red cells during the early weeks of cholesterol feeding was normal at a time when red cell enrichment with cholesterol had already been established. This is analogous to the observations in patients in whom it was found that the osmotic fragility of red cells is normal despite their added cholesterol (7). Using 51Cr-labeled red cells in patients with spur cell anemia, it has been shown that normal red cells initially become resistant to osmotic lysis after exposure to the cholesterol-enriched environment in vivo, but within 24 hr begin to return to a normal osmotic fragility (7). In the absence of the spleen, red cells in spur cell anemia retain their decreased osmotic fragility (36). From these two observations it was concluded that the process of addition of cholesterol to red cell membranes both in vivo and in vitro is associated with an expansion of membrane surface area and a decrease in osmotic fragility. In the presence of the spleen in vivo, these red cells undergo a secondary modification of membrane architecture characterized by further changes in red cell morphology and the loss of surface area, thereby reestablishing an osmotic fragility that is within the normal range (7, 36). Indeed, removal of the excess cholesterol from such cells results in spherocytic, osmotically fragile red cells (7).

The delayed onset of hemolysis in guinea pigs and rabbits, as well as the delayed onset of anemia in dogs herein, suggests that some factors beyond cholesterol enrichment of red cell membranes alone may be crucial for the development of anemia. To what extent are progressive liver disease and splenic congestion these factors? Liver disease in man is known to have two independent effects on anemia (37). First is decreased red cell production, and second is premature red cell destruction in the spleen. In man, congestive splenomegaly alone is capable of modifying red cell membranes and causing a mild degree of cell destruction (38). It is unknown whether, in any of the animal species fed cholesterol-enriched diets, portal cirrhosis induces portal hypertension and splenic congestion and, if so, whether it induces a hemolytic component. Red cell survival was not directly determined in the present study. The small but significant reticulocytosis in beagles may reflect a mild hemolytic component. However, the absence of reticulocytosis in greyhounds suggests that anemia in this species was due predominantly to underproduction.

In both species, the falling hematocrit observed was coincident with an increase in osmotic fragility, suggesting that red cell surface area decreased. This suggests that the changes in membrane cholesterol and fluidity during the initial 6 weeks of dietary cholesterol were insufficient in magnitude to lead...
to red cell damage in vivo. In contrast, the increment in both which occurred between 6 and 12 weeks may have created a magnitude of membrane structural disorder that was sufficient to be perceived as abnormal, thus leading to a loss of red cell surface area and premature red cell destruction. In man, the predominant organ responsible for the destruction of spur cells is the spleen. Whether or not this is the case in dogs is unknown.

This report represents the first demonstration of spur cell anemia in dogs induced by cholesterol feeding. This process is reversible, and, indeed, was acquired and reversed on two successive occasions in a single animal. This study serves to emphasize the importance of cholesterol homeostasis for the maintenance of normal red cells in vivo.

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