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Richard A. Cooper, James H. Jandl

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Research Article

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Bile Salts and Cholesterol in the Pathogenesis of Target Cells in Obstructive Jaundice

RICHARD A. COOPER and JAMES H. JANDL

From the Thorndike Memorial Laboratory and 2nd and 4th (Harvard) Medical Services, Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston, Massachusetts 02118

ABSTRACT Free cholesterol is in rapid equilibrium between serum lipoproteins and red cells. The level of red cell cholesterol is influenced by bile salts, which shift the serum/cell partition of free cholesterol to the cell phase and which inhibit the cholesterol-esterifying mechanism. During incubation in normal serum possessing an active cholesterol-esterifying mechanism, red cells lose cholesterol and surface area and thereby become more spheroidal and less resistant to osmotic lysis. When exposed to serum from patients with obstructive jaundice or to normal serum with added bile salts, red cells accumulate cholesterol and increase their surface area, thereby acquiring a flattened shape and an increased resistance to osmotic lysis. The described gains and losses of red cell cholesterol and surface area do not involve metabolic injury and occur with no significant change in phospholipid content.

The red cells of patients with obstructive jaundice are flat and osmotically resistant and have an increased cholesterol : phospholipid ratio. When transfused into normal subjects these "target cells" rapidly lose their osmotic resistance. Similarly, normal cells acquire osmotic resistance in the circulation of patients with obstructive jaundice. These reversible changes in shape occur with half-times of about 9 and 24 hr, respectively, and occur without impairing cell viability.

These studies indicate that the red cell membrane accumulates cholesterol in obstructive jaundice as a consequence of the elevated levels of bile salts. The resulting increment in red cell surface area is responsible for the physical properties and appearance of target cells. These observations substantiate Murphy's findings in vitro indicating that cholesterol is an important determinant of red cell shape and that its content in the cell membrane may vary independently from the phospholipids. Presumably any process or disorder affecting cholesterol exchange in vivo is capable of critically modifying the shape and behavior of red cells.

INTRODUCTION

An increased resistance of red cells to osmotic lysis is frequently encountered in patients with hepatitis (1), particularly during the obstructive phase, and is almost always observed in patients with extrahepatic biliary tract obstruction. In obstructive jaundice there is usually a symmetrical, and often a marked, shift in the osmotic fragility curve, which indicates a homogeneous population of osmotically resistant cells. Lesser increments in osmotic resistance, complicated by cellular heterogeneity, are encountered in patients with chronic liver disease accompanied by an obstructive component (2). The osmotic resistance of red cells in obstructive jaundice is attributable to a characteristic increase in the surface-to-volume ratio (3). Affected cells have a normal volume but an increased surface area, which gives them a broad,

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Dr. Cooper is a Research Fellow, Harvard Medical School.

Dr. Jandl is an Associate Professor of Medicine, Harvard Medical School.

Address requests for reprints to Dr. Richard A. Cooper, Thorndike Memorial Laboratory, Boston City Hospital, Boston, Mass. 02118.

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flat configuration. On stained smears the drying pattern creates an appearance described by the term target cell (4).

That osmotic resistance can be acquired by mature red cells was suggested by Berk (5) on the basis of observations in patients with obstructive jaundice who had been transfused. Later in a preliminary report Harris and Schilling (6) substantiated this in a study which made use of differential agglutination techniques for separating out normal red cells after their transfusion into jaundiced patients.

Little is known of the normal mechanisms responsible for the shape of red cells or, accordingly, of the abnormality that engenders the flattened shape of target cells. It is known that red cell survival is unaffected by obstructive jaundice per se (7) and that the shape-change is probably reversible, judging from the fact that the osmotic fragility pattern reverts to normal within 2 wk after surgical relief of biliary obstruction (5, 6) or after the subsidence of hepatitis (8). Although increased osmotic resistance has not correlated well with serum levels of total cholesterol (9, 10) or bilirubin (8, 9, 11), a relationship to bile salt retention was suggested in 1931 by Geill (8). Attempts to produce osmotically resistant cells in vitro by incubating normal red cells in the sera of patients with obstructive jaundice have been unsuccessful (6); however, such incubations are complicated and opposed by the increased osmotic fragility which results from metabolic injury (12). Recently Murphy (13) incubated red cells under conditions that minimized metabolic damage. Nonetheless, when suspended in normal fresh serum, red cells showed a progressive increase in osmotic fragility. This was not observed, however, in cells suspended in heated serum. The increased osmotic fragility that developed over a period of many hours in serum was associated with a loss of red cell cholesterol, but not phospholipid, and with a decline in serum free cholesterol due to its esterification by the heat-labile serum enzyme, transesterase.

Cholesterol is confined to the membrane of the red cell (14), where it exists almost entirely in the free (unesterified) form, comprising about 30% of the membrane lipids (15). Unlike the several phospholipids, which turn over or exchange with serum lipids slowly or not at all (16), red cell cholesterol is in a rapid exchange equilibrium with the free cholesterol of serum, wherein it exists bound to lipoproteins (17). Murphy has provided evidence that the distribution of free cholesterol in the cell membrane may be responsible for the cell's biconcave shape (18). The conclusion that spherocytosis can be induced in vitro by a selective depletion of cholesterol had not been confirmed before the present studies (19), and, indeed, various studies by others (20, 21) of membrane lipids during prolonged incubation of blood in vitro have reported only a process of proportional or "symmetrical" loss of lipids, in which red cells lose both cholesterol and phospholipids in equivalent amounts.

The present studies of the pathogenesis of target cells in obstructive jaundice were undertaken because of the potentially great importance of a process that allows continued modification of cell membrane composition and thereby of shape and possibly function. Several observations suggested the hypothesis that target cells may represent a specific increase in membrane cholesterol: (*a*) the serum levels of free cholesterol tend to be elevated in biliary obstruction and there is at least one report (9) that the increased osmotic resistance in red cells from jaundiced patients (with hepatitis) is associated with an increase in red cell cholesterol; (*b*) transesterase activity is decreased in the sera of patients with obstructive jaundice (22) and that of normal serum is inhibited by bile salts (23, 24), which are characteristically elevated in obstructive jaundice; and (*c*) with respect to the surface area/volume ratio, the potential "hemolytic volume," and the osmotic resistance, target cells are essentially the opposite of spherocytes which can be produced in vitro by cholesterol elution (13). The following investigations of this hypothesis have been reported in part in preliminary form (19).

METHODS

Incubation in vitro. Blood from normal subjects was defibrinated with glass beads and the red cells were washed three times in isotonic phosphate buffer, pH 7.4, with added glucose (Na, 167 mEq/liter; K, 20 mEq/liter; PO_4 120 mEq/liter; and glucose, 150 mg/100 ml) to remove serum and buffy coat. For most studies, washed red cells were resuspended in buffered serum (2 parts buffer and 1 part serum) at a cell concentration of only 2%, in order to minimize alterations in pH or substrate

concentrations during incubation. In studies requiring direct measurement of maximum hemolytic volume and for measurements of serum–red cell cholesterol balance, both requiring higher cell concentrations, the washed red cells were resuspended in serum without added buffer but with added glucose (400 mg/100 ml) at a red cell concentration of 35%. Penicillin, 500 U/ml, and streptomycin, 250 ug/ml, were added to the red cell suspensions. Incubations were performed in stoppered glass vials at 37°C in a Dubnoff metabolic shaker at 110 oscillations/min for 24 hr (hematocrit 2%) or 12 hr (hematocrit 35%). pH levels were 7.3–7.5 at hematocrit 2% and 7.1–7.5 at hematocrit 35%.

Osmotic fragility and estimated cell surface area. From duplicate incubations 0.2 ml of cell suspension was added to 4.0 ml aliquots of saline in each of a series of tubes containing a graded sequence of concentrations of NaCl. The NaCl concentrations of the solutions of oven-dried NaCl were verified by direct measurement of the Na concentration. After the addition of red cells, each tube was mixed immediately and centrifuged within a period of from 2–3 min after admixture. Per cent hemolysis was measured at 540 mμ and the osmotic fragility so obtained was corrected for the effect of pH and "tonicity" (25). The spherical volume of red cells was calculated on the basis of the volume increment at their mean osmotic fragility, according to Castle and Daland (26). This calculation depends on predictable changes in cell volume in hypotonic salt solutions of known tonicity. It is based on the Boyle-van't Hoff law which considers the red cell as a perfect osmometer. The formulation of Castle and Daland is in good agreement with the more recent findings of Hoffman, Eden, Barr, and Bedell (27) and Savitz, Sidel, and Solomon (28). In the present study it has been used to compare the spherical volume of a single population of red cells before and after incubation. Since this volume, the maximum volume attainable before hemolysis, depends on (*a*) the intracellular content of osmotically active material and (*b*) the surface area of the limiting cell membrane, it serves to reflect the latter only if there has been no change in the former. Thus its use requires that the red cell population measured be a single population distributed about a mean, that the cation content be the same in all samples of red cells measured, and that the red cells be exposed to hypotonic solutions for a period of time shorter than that which would itself cause an appreciable change in fragility due to cation changes. All of these conditions were met. The surface area of red cells after incubation relative to their surface area before incubation was calculated from their spherical volumes. The error of duplicate measurements of the osmotic fragility values from which this calculation was made imparted a standard error of 0.5% to the expression of surface area.

Red cell and serum lipids. The red cells of quadruplicate incubation vials were quantitatively transferred to extraction tubes and the serum was removed by three saline washes. The cell button was twice extracted with isopropanol and chloroform (29). Cholesterol was measured by the method of Zlatkis, Zak, and Boyle (30) and lipid phosphorus by Böttcher, Gent, and Pries' modification (31) of the Fiske-Subbarow reaction (32). Serum free cholesterol was determined by the method of Brown, Zlatkis, Zak, and Boyle (33). Standard errors of extraction and measurement combined (95% confidence limits) are: red cell cholesterol ± 1.8%, red cell lipid phosphorus ± 1.3%, and serum free cholesterol ± 1.4%. Red cells were enumerated in the Coulter model B electronic counter.

For measurement of red cell K and volume the contents of triplicate vials were quantitatively transferred to graduated Constable tubes, centrifuged, and the meniscus washed with isotonic NaCl. The red cell button was lysed with 1.0 ml of distilled water and K measured in a flame photometer.[1] Diameters of red cells suspended in incubation medium were made with a calibrated eyepiece micrometer.[2] Serum bile salts were determined by gas-liquid chromatography. (34).

^{51}Cr osmotic fragility (35). 30-ml specimens of whole blood from patients with surgically proven extrahepatic biliary tract obstruction or normal subjects were collected into sterile acid citrate dextrose, after which 300 μc of ^{51}Cr-labeled sodium chromate[3] was added. After 20 min at room temperature the cells were centrifuged once and resuspended in sterile saline for injection into a compatible recipient. At intervals thereafter survival of the labeled cells was measured and their osmotic fragility as well as that of the recipient's red cells was determined as follows: 0.5 ml of oxalated whole blood was added to a series of tubes containing 2.5 ml of hypotonic sodium chloride solutions. Per cent hemolysis of the transfused cells was measured as per cent radioactivity released into the supernatant. Per cent hemolysis of the recipients' own cells was measured as per cent hemoglobin released. Osmotic fragilities were corrected for tonicity of the added blood and for pH according to Emerson, Shen, Ham, Fleming, and Castle (25).

Patients. Patients were chosen for study on the basis of having: (*a*) relatively stable, uncomplicated, obstructive jaundice; (*b*) a uniform population of target cells on blood smear and a striking increase in osmotic resistance; and (*c*) no other significant hematologic abnormality. All patients or subjects who underwent ^{51}Cr–red cell survival studies gave their full consent after being fully informed of the nature of the studies and of their potential risks. In two instances the red cell donors were jaundiced; both were established in advance to be affected by extrahepatic biliary tract obstruction, as verified by surgical exploration and liver biopsy. The protocol followed in their study was approved by the review committee of the Clinical Center Ward of the Thorndike Memorial Laboratory.

Reagents. Sodium taurocholate[4] was a mixture of bile salts, greater than 98% conjugated, consisting of 83%

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[1] Instrumentation Laboratory, Inc., Watertown, Mass.

[2] Bausch & Lomb Incorporated, Rochester, N. Y.

[3] Available as Rachromate from Abbott Laboratories, North Chicago, Ill.

[4] Available from Sigma Chemical Co., St. Louis, Mo.

cholate, 16% deoxycholate, and 1% chenodeoxycholate.[5] Purity of other bile salts was stated by the supplier[4] to be: glycocholate > 95%; cholate > 99%; deoxycholate > 95%.

Statistics. Slopes were calculated by the method of least squares. Significance of the difference between slopes and standard errors, expressed as 95% confidence limits, were calculated by an analysis of variance (36).

RESULTS

Studies in vivo. When normal red cells labeled with ^{51}Cr were transfused into a compatible normal recipient, they manifested no change in osmotic fragility during the 32 day period of observation. However, when cells from four normal donors were transfused into four patients with obstructive jaundice, the osmotic fragility curves of the labeled cells became progressively shifted toward those of the recipients. Fig. 1 depicts one such transfusion in which the osmotic fragility curve of the normal transfused cells had shifted halfway toward that of the resistant cells of the patient in about 24 hr. In the converse experiment red cells from two patients with surgically proven obstructive jaundice and target cells were labeled with ^{51}Cr and transfused into two normal recipients, with similar results in each patient. In the study depicted in Fig. 2, the transfused target cells rapidly lost their abnormal resistance to osmotic lysis and acquired the osmotic characteristics of the recipient's normal cells.

Figure 3 summarizes the results of six separate transfusion studies and shows the rates at which the mean osmotic fragility of the transfused cells approached that of the recipients' cells. The ^{51}Cr half-life of the transfused cells was greater than

[5] This analysis was kindly performed by Dr. W. G. Hardison.

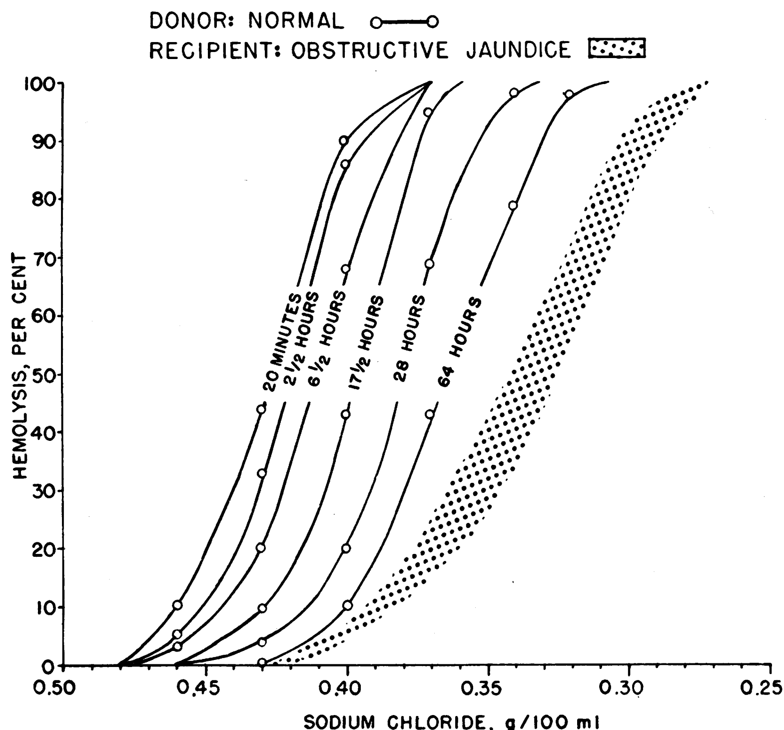

FIGURE 1 Changes in the osmotic fragility of normal red cells after transfusion into a patient with obstructive jaundice. Normal red cells became progressively more resistant to osmotic lysis after transfusion into a patient with jaundice caused by extrahepatic obstruction. This change was halfway complete in about 24 hr. In this figure and in Figs. 2 and 3, each osmotic fragility curve of the donor cells was determined with ^{51}Cr and is identified in terms of the time interval after infusion. The range of the osmotic fragility curves of the recipients during the entire period of study is depicted by the stippled areas.

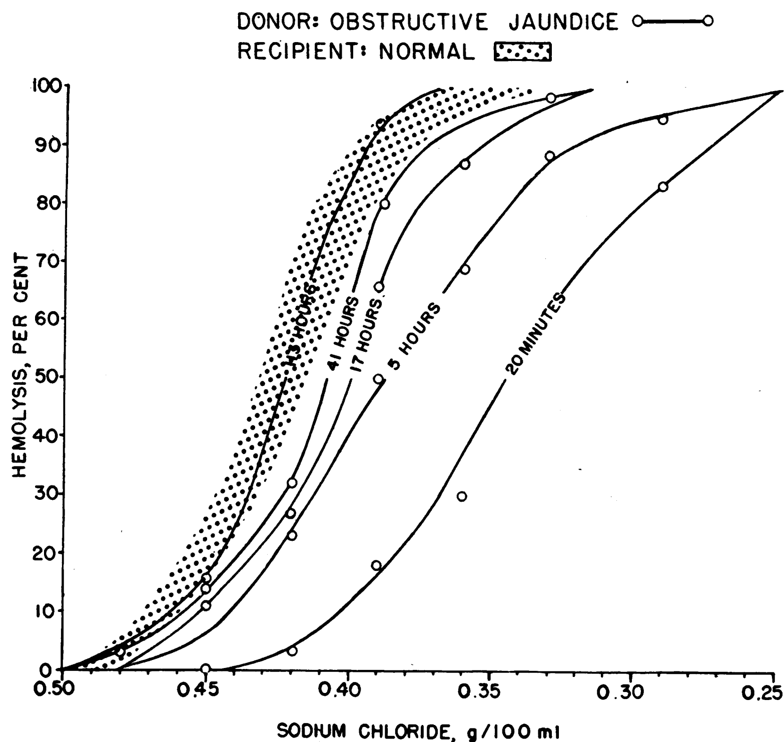

FIGURE 2 Changes in the osmotic fragility of red cells from a patient with obstructive jaundice after their transfusion into a normal recipient. Osmotically resistant cells from a patient with target cells progressed steadily toward a normal osmotic fragility after transfusion into a normal subject.

20 days in five of the six studies; however, in one, involving the transfusion of red cells from a jaundiced donor to a normal recipient, the ^{51}Cr half-life was 5 days. The unexplained shortening of survival in this latter transfusion occurred at a rate slower than the change in osmotic fragility. Therefore, in five studies, and probably in the sixth, the observed osmotic fragility changes represent a transformation of the cells transfused, rather than the selective survival of a more or less resistant minor population of cells. The acquisition of increased osmotic resistance in vivo appeared to occur as a zero order function of time, 50% of the change occurring in approximately 24

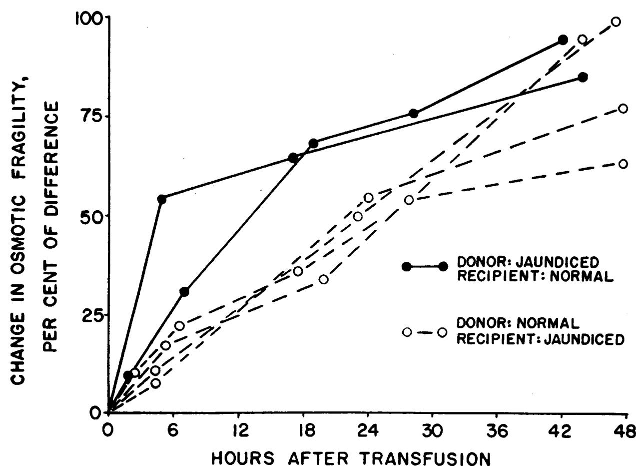

FIGURE 3 Rate of change in osmotic fragility in vivo. The differences between the mean osmotic fragility of ^{51}Cr-labeled donor red cells and the unlabeled red cells of the recipient at various times after transfusion is plotted as per cent of the initial difference. The ^{51}Cr half-life of the transfused red cells was greater than 20 days in all but one instance, depicted by the lower of the two uninterrupted lines as of 6 hr, in which the ^{51}Cr half-life was 5 days.

hr. The process by which target cells lost their resistance to osmotic lysis took place more rapidly, with 50% of the transition complete in 9 hr. No firm conclusion as to kinetics is possible, the rate at which osmotic resistance was lost correlating equally well with zero and first order kinetics.

Effect of normal sera on normal red cells in vitro. Incubations were carried out in a manner which minimized metabolic damage to the red cells. This was shown by the absence of a change in the osmotic fragility, in all experiments, and in volume and K content, in initial experiments, when normal red cells were incubated for 24 hr at a cell concentration of 2% in a serum-buffer-glucose medium using normal serum previously heated to 56°C for 30 min. Similarly there was no change in osmotic fragility when 6% human albumin was substituted for serum in this medium. However, when the medium contained fresh (unheated) normal serum, normal red cells underwent an increase in osmotic fragility and became more spheroidal (Fig. 4) as reported by Murphy (13). This occurred without a change in volume and thus resulted from a loss of membrane surface area. Associated with the loss of surface area was both a proportional loss of red cell cholesterol (Fig. 5) and a proportional fall in the serum concentration of free cholesterol (Fig. 6). When the transesterase activity of normal sera was destroyed by heating at 56°C for 30 min before incubation, there was no fall in serum free cholesterol levels during incubation, and red cell cholesterol and surface area remained unchanged (Fig. 6). Despite the close correlation that existed when changes in red cell surface area were plotted against changes in serum free cholesterol concentration in normal sera, surface area was not affected by the actual concentration of cholesterol per se; thus there was no change in surface area when normal red cells were incubated with seven homologous normal sera that differed widely in their concentrations of free cholesterol, provided the esterifying activity had been inactivated by heating. Moreover, after incubation in fresh (unheated) homologous sera there was a poor correlation between cell surface area and serum free cholesterol concentration per se; incubation of normal red cells in sera from patients with familial

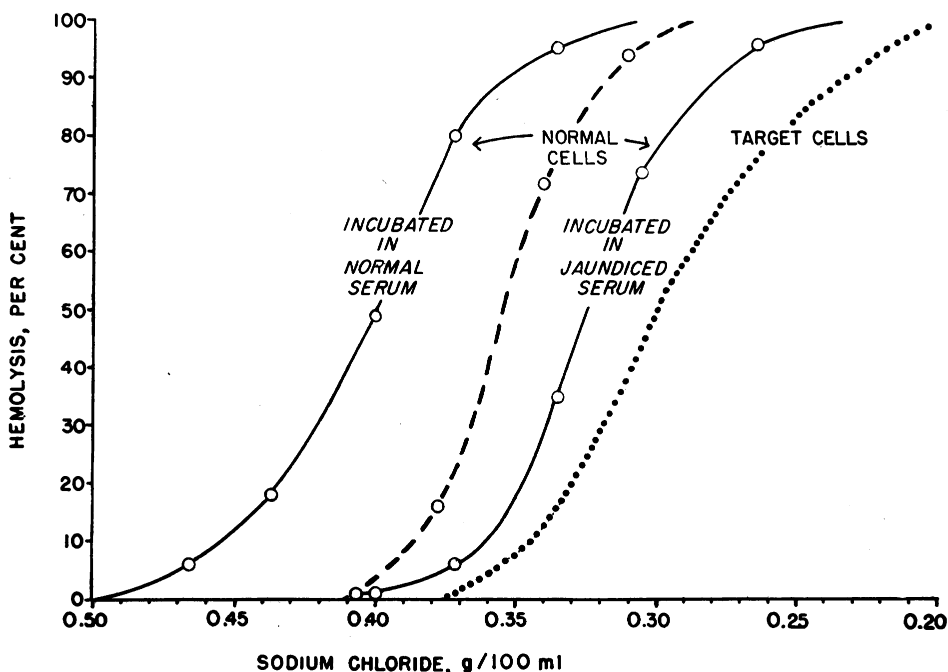

FIGURE 4 The effect of normal and jaundiced sera on the osmotic fragility of normal red cells during incubation at 37°C for 24 hr. Whereas normal red cells became osmotically fragile in normal serum, they became osmotically resistant in serum from a patient with obstructive jaundice. The interrupted line represents osmotic fragility before incubation. The dotted line represents the osmotic fragility of red cells (target cells) from the jaundiced patient.

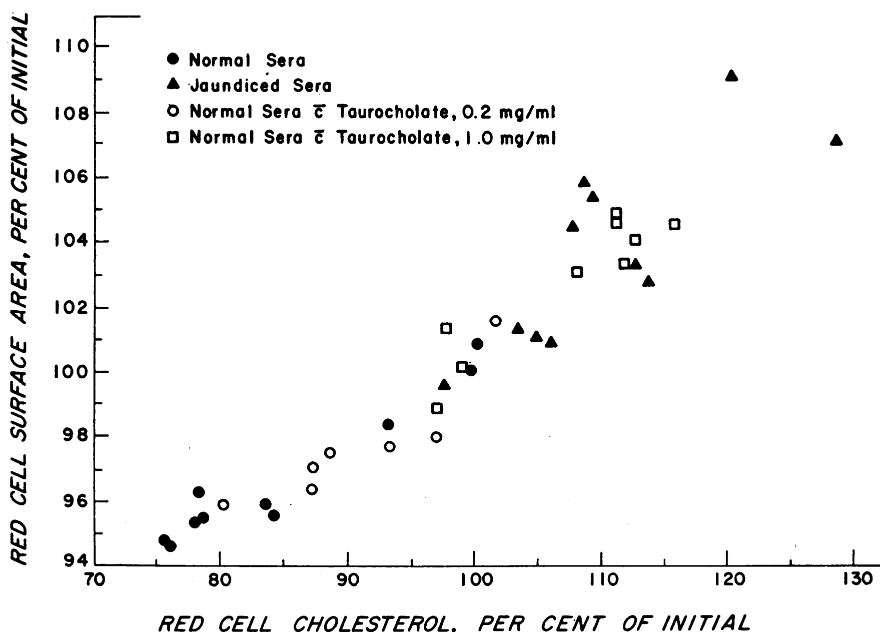

FIGURE 5 Relationship between the cholesterol content and surface area of red cells after incubation in various sera at 37°C for 24 hr. Both are expressed as per cent of the initial values before incubation. A direct correlation was seen under all of the conditions examined.

hypercholesterolemia resulted in a slight decrease in surface area, despite the fact that the free cholesterol concentration of these sera was more than twice that of the autologous normal serum. However, when the free cholesterol concentrations of these homologous sera were expressed as a per cent of the concentration of free cholesterol existing in each before incubation, i.e. as the change in free cholesterol level, there was close correlation with red cell surface area (Fig. 6). Thus in both normal and hypercholesterolemic sera it appears to be the relative decrease in serum free cholesterol during incubation, rather than its absolute concentration, which correlates with red cell cholesterol and surface area.

Effect of jaundiced sera on normal red cells in vitro. In contrast to the loss of surface area and increase in osmotic fragility regularly seen after red cells have been incubated in fresh normal serum, incubation of normal red cells in sera from patients with biliary tract obstruction, who were jaundiced and had target cells, resulted invariably in a gain of surface area and an increased resistance to osmotic lysis (Fig. 4). This gain in surface area was associated with a proportional increment in red cell cholesterol (Fig. 5). Although the serum concentration of free cholesterol in patients with obstructive jaundice was usually elevated, frequently to a marked degree, there was no systematic relationship between the serum concentration of free cholesterol and the red cell surface area after incubation. Nevertheless, incubations in jaundiced sera induced increments, rather than decrements, in the surface area and cholesterol content of normal cells (Figs. 5 and 6). Moreover, the surface area of normal red cells was greater after incubation in all 11 of the fresh jaundiced sera studied, whereas this occurred with none of the nonjaundiced sera despite and overlap in the percentage of cholesterol esterification (Fig. 6), and it occurred also with jaundiced sera heated to 56°C for 30 min. Therefore, the unique effect of jaundiced sera cannot be attributed solely to suppression of cholesterol esterification.

Effect of bile salts on normal red cells in vitro. Ponder (37) observed that bile salts added to washed red cells in a saline suspension cause hemolysis, but that this does not occur in the presence of serum or albumin. Under the conditions of the present study taurocholate caused no hemolysis unless its concentration exceeded about 4.0 mg/ml of serum.

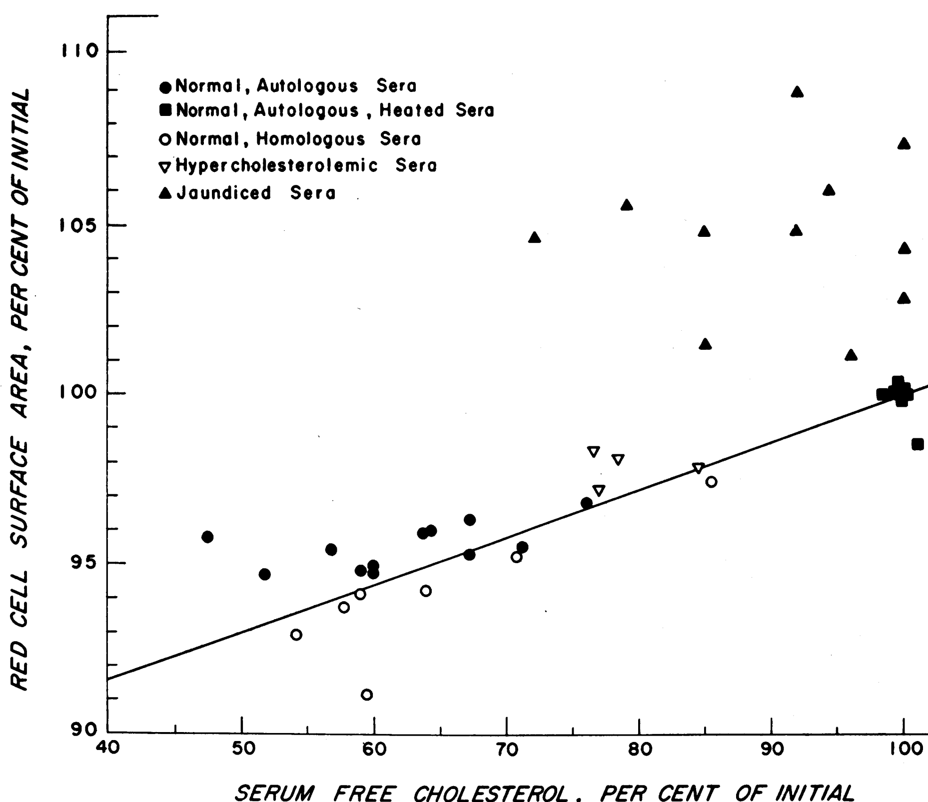

FIGURE 6 Relationship between changes in serum levels of free cholesterol and in red cell surface areas after incubation of normal red cells in various sera at 37°C for 24 hr. In sera from normal subjects and from subjects with familial hypercholesterolemia, changes in red cell surface area correlated closely with changes in serum free cholesterol concentration, expressed as per cent of its concentration before incubation. The slope expressing this correlation was plotted by the method of least squares. In all 11 sera from patients with obstructive jaundice and target cells the surface area of normal red cells after incubation was greater (regardless of the extent of change in serum cholesterol level) than in nonjaundiced sera.

The calculated surface area of normal red cells after incubation for 24 hr in normal sera to which taurocholate had been added was greater than that of cells after parallel incubation in the same normal serum having an equal concentration of free cholesterol but without added bile salts (Fig. 7). As in the previous incubations, the increase in surface area in cells incubated with taurocholate-serum was accompanied by a proportional increase in red cell cholesterol (Fig. 5). Results similar to those with taurocholate were also obtained with glycocholate, cholate, or deoxycholate at equal concentrations (by weight). However, there was no change in surface area when red cells were incubated in a medium in which serum was replaced by 6% albumin and contained cholate in serial concentrations up to that which was hemolytic.

After incubation in normal serum containing taurocholate, 1.0 mg/ml, normal red cells were increased in surface area as determined from their maximum volume in hypotonic saline (38) and as calculated from their osmotic fragility curve (26). They were also increased in diameter when examined directly in an isotonic suspension using an optical micrometer. The mean cell diameter $\pm$ standard error (95% confidence limit) of red cells after incubation in heated normal serum was $7.47 \pm 0.27\ \mu$; after incubation in heated normal serum containing taurocholate, the mean diameter was $8.14 \pm 0.47\ \mu$ ($P < 0.01$).

Although red cells gained cholesterol when incubated at a cell concentration of 2% in normal serum containing taurocholate, the amount of cholesterol transferred from serum to cells was too

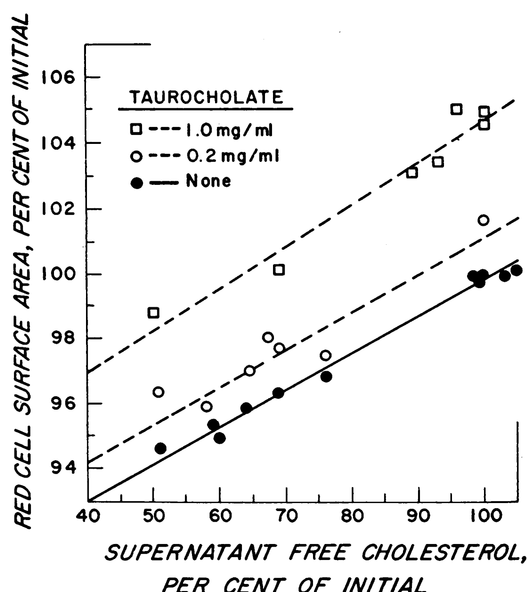

FIGURE 7 Influence of taurocholate on red cell surface area after incubation of normal red cells in normal serum at 37°C for 24 hr. At each serum concentration of free cholesterol the red cell surface area is directly related to the concentration of taurocholate. The intercept of each slope differs from the others statistically ($P < 0.01$).

small to be measurable as a decline in serum free cholesterol. Therefore, incubation conditions were altered so as to increase the ratio of cells/serum. As metabolic injury to red cells occurs much sooner with the more concentrated suspensions, it was necessary to terminate the incubation period sooner (at 12 hr) and accordingly to employ a higher level of bile salts. At a cell concentration of 30–35% in normal serum which had been heated to 56°C and to which was added taurocholate, 2.0 mg/ml, a rise in red cell cholesterol was accompanied by a reciprocal fall in the serum level of free cholesterol (Table I). The cumulative increase in cell cholesterol and surface area occurred as a linear arithmetic function of time ($t_{\frac{1}{2}} = 6$ hr), there being no immediate shift in the cholesterol partition. Thus it appears that bile salts cause an increase in red cell surface area by mediating a gradual transfer of free cholesterol from serum to the cell membrane. In none of the experiments described above, regardless of the gain or loss of red cell cholesterol, was there a change in red cell phospholipid content in excess of the error of the method.

Cholesterol esterification in vitro. The esterification of cholesterol in incubation mixtures which contained red cells at a concentration of 2% and normal serum was 200 ± 20 μg/ml of serum in 24 hr. With jaundiced sera, despite higher levels of free cholesterol, esterification averaged only 100 ± 42 μg/ml of serum in 24 hr. Cholesterol esterification in normal serum was inhibited by the addition of taurocholate in proportion to its concentration. The per cent inhibition was 8.3 ± 4.6% at a taurocholate concentration of 0.2 mg/ml; 28.3 ± 11.9% at 0.5 mg/ml; and 78.8 ± 3.8% at 1.0 mg/ml. The inhibition of esterification of cholesterol in jaundiced sera or in normal sera containing added taurocholate prevented the net loss of red cell cholesterol during incubation, thus tending to maintain the membrane composition, surface area, and, accordingly, the shape of the cells. Cholesterol esterification occurred equally well in normal serum incubated in glass or plastic vessels and in plasma anticoagulated with ethylenediaminetetraacetate (EDTA).

Red cell lipids and serum bile salts in obstructive jaundice. In 16 normal subjects red cell cholesterol was 13.4 ± 0.4 μg/10^8 cells and lipid phosphorus was 1.08 ± 0.04μg/10^8 cells. The cholesterol : lipid phosphorus ratio was 12.5 : 1. These values fall within the range of values for red cell

TABLE I

*Effect of Taurocholate on the Cholesterol Partition between Serum and Red Cells**

	Content of free cholesterol		
	After incubation in heated serum	After incubation in heated serum with taurocholate	Change with taurocholate
	μg/ml of suspension		
Expt. A			
Whole suspension	871	872	+ 1
RBC	528	570	+42
Serum	343	302	−41
Expt. B			
Whole suspension	765	770	+ 5
RBC	438	490	+52
Serum	327	280	−47

* Normal red cells were incubated for 12 hr at 37°C at a cell concentration of 30–35% in normal serum, previously heated to 56°C for 30. min, containing added glucose at a concentration of 400 mg/100 ml. Under these experimental conditions the cholesterol content of red cells and of heated serum after incubation remain identical to values measured before incubation. Sodium taurocholate was added to this serum medium so as to achieve a final concentration of 2.0 mg/ml.

lipids previously reported by several observers (15). The cholesterol content of red cells from six patients with obstructive jaundice was 17.9 ± 4.3 $\mu g/10^8$ cells; lipid phosphorus was 1.26 ± 0.23 $\mu g/10^8$ cells. The cholesterol: lipid phosphorus ratio was 14.5:1. The red cells from patients with obstructive jaundice showed an average increase above normal for cholesterol of +34% ($P < 0.001$), for phosphorus of +17% ($P < 0.05$), and for the cholesterol: lipid phosphorus ratio of + 16% ($P = <0.01$).

The serum concentration of bile salts in four patients with obstructive jaundice ranged from 4.8 to 19.5 $\mu g/ml$.[5]

DISCUSSION

These studies demonstrate that mature red cells are capable of gaining and losing membrane cholesterol as a specific and reversible process. Changes in the cholesterol content of red cells are accompanied by proportional changes in their surface area and thus in their shape, appearance, and physical capacity for water and electrolytes. These associated losses or gains in the cholesterol content, surface area, osmotic resistance, and potential (hemolytic) volume of red cells do not involve metabolic damage or changes in actual cell volume. The observation that cholesterol alone is lost from red cells during incubation in normal serum, provided metabolic injury is avoided, confirms previous studies by Murphy (13). That cholesterol loss is unaccompanied by changes in red cell phospholipids indicates that this process in viable red cells differs from the phenomena studied by Reed and Swisher (20) and Jacob (21) in which proportional or "symmetrical" losses of cholesterol and phospholipid occurred, possibly reflecting membrane fragmentation, in cells during prolonged incubation and metabloic injury. The present observations in vitro and in vivo substantiate the hypothesis that the target cell of obstructive jaundice reflects a process whereby cholesterol selectively accumulates in the red cell membranes. This accumulation appears to be favored by two general factors: (*a*) the serum level of free cholesterol, expressed in terms of the saturation of its binding proteins, and (*b*) the serum level of bile salts, which influence the serum/cell partition of free cholesterol. As cholesterol accumulates on the red cell membrane, the surface area is extended proportionally. Since red cell volume and cation content do not change under the conditions of incubation used, this increase in surface area causes red cells to assume a flattened shape and allows a greater increment in volume during hypotonic swelling, rendering the cells osmotically "resistant." Target cells are the morphological expression of an increased surface-to-volume ratio and can result from either an increase in surface while volume remains constant, as in the present study, or a decrease in volume while surface area remains constant, as in hypertonic serum (39).

The progressive increase in osmotic resistance of normal red cells during incubation in jaundiced sera in vitro mimics similar increases in vivo after normal red cells are transfused into recipients with obstructive jaundice. It is inferred, therefore, that the gain in cholesterol (but not in phospholipid) that accompanied the acquisition of osmotic resistance in vitro also took place as normal red cells became resistant in vivo. Supporting this inference is the finding that the red cells from our patients with obstructive jaundice were disproportionately high in cholesterol (+ 34%), although there was a lesser increase in phospholipid (+ 16%) as well. Neerhout (40, and footnote 6), studying a diverse group of 24 patients with varying degrees of cellular and obstructive liver disease, many of whom had anemia and macrocytosis, also recorded a disproportionate increase in red cell cholesterol (+ 26%) as compared with phospholipid (+ 12%). A portion of the increases in this heterogeneous group of patients was due to the "symmetrical" rise in both phospholipid and cholesterol due to macrocytosis, i.e. red cells increased both in volume and in surface area, and reticulocytosis. Selective, or "asymmetrical," increments in red cell cholesterol are expressed by the cholesterol/phospholipid phosphorus ratio: in Neerhout's unselected series of patients with liver disease this ratio was increased on the average by 11% over normal; in our patients with obstructive jaundice the increase averaged 16%. The selective increments in cholesterol observed in red cells in vitro and the predominance of the cholesterol increases found in the red cells of jaundiced patients indicates either that cholesterol gain is the primary event in obstructive jaundice, with phospholipid acquisition being a variable, secondary event, or

[6] Neerhout, R. C. Personal communication.

that both moieties may tend to accumulate, cholesterol accumulating more rapidly because of its far greater rate of turnover (see below). In either event changes in cholesterol clearly do not necessitate equivalent changes in phospholipid.

The lack of any gain in red cell lipids in familial hypercholesterolemia, despite serum lipid levels as high or higher than occur in obstructive jaundice (10, 40, 41), emphasizes the importance of factors apart from high levels of serum cholesterol per se. The present findings indicate that bile salts when added to normal serum in vitro affect the cholesterol levels of red cells in two distinct ways. First, as reported by others (23, 24), bile salts inhibit the activity of the serum-esterifying system; this in turn inhibits the depletion of free cholesterol from red cells during incubation (see below). Secondly, bile salts induce a shift in the red cell/serum partition for cholesterol, resulting gradually in supranormal levels of cholesterol in the cells. The close association of the target cell phenomenon with disorders characterized by bile salt retention provides circumstantial support for the conclusion that the increase of cholesterol and surface area in the patients' red cells is similarly mediated by bile salts. It should be noted that the concentrations of bile salts required to produce target cells in vitro (100–1000 μg/ml) exceed those in peripheral blood from patients with obstructive jaundice, wherein the serum levels usually fall within the range of 5–100 μg/ml (34, 42). This difference does not represent a serious discrepancy, however, since the response of red cells to the 12–24 hr periods of exposure to serum containing bile salts as observed in vitro only partially reflects the process in vivo, which is sustained for 48–72 hr. Moreover, in contrast to the situation during incubation in vitro where transfer of cholesterol from serum to red cells results in unsaturation of the serum binding sites, plasma free cholesterol in vivo would tend to remain saturated through equilibration with the total body pool of free cholesterol (43–45).

The selective depletion of red cell cholesterol during incubation in serum results from depletion of the serum free cholesterol, with which the cells are in equilibrium, through the action of the serum enzyme, transesterase. This occurs in plasma anticoagulated with EDTA as well as serum and in plastic as well as glass. Esterification of free cholesterol causes unsaturation of the lipoprotein moieties which bind it. The availability of these lipoprotein sites for recombination with free cholesterol derived from red cells was demonstrated by Murphy (13) who showed a return of serum free cholesterol levels toward normal when red cells were added to serum that had been depleted of free cholesterol by previous esterification. Presumably it is the relative saturation of these lipoprotein-binding sites rather than the absolute concentration of serum-free cholesterol which determines the per cent saturation of red cell cholesterol. This view is consistent with the finding of normal or only slightly reduced levels of cholesterol in red cells from patients with Tangier disease or acanthocytosis, diseases associated with a marked decrease in serum free cholesterol due to deficiencies of α- and β-lipoproteins, respectively (46–49). The selective loss of cholesterol from the red cell membrane alters the cell in a manner directly opposite that observed during the formation of target cells: the depleted membrane is proportionally diminished in surface area and therefore the cell assumes a more spheroidal shape. Both cholesterol and shape can be restored to the red cell in vitro (13, 50) and in vivo (50).

It has not definitely been established in vivo whether cholesterol esters are mainly formed from free cholesterol in plasma through the action of plasma (serum) transesterase or whether they are mainly formed in the liver and incorporated there into lipoproteins (51–53). However, Norum (54) has recently described three siblings who lack serum transesterase activity but who retain the capacity to esterify cholesterol in the intestine. They have elevated serum cholesterol, only small amounts of which is esterified. Red cells from these patients are described by the author [7] as having the appearance of target cells and containing increased amounts of free cholesterol but normal amounts of phospholipid. Thus it appears that diminished activity of transesterase, as also occurs in obstructive jaundice (22), promotes the accumulation of red cell cholesterol. Conversely, the studies in vitro indicate a significant potential for esterification of free cholesterol and loss of red cell cholesterol in any situation in vivo wherein conditions of plasma skimming and erythroconcentration may exist, as in the spleen. The sphering

[7] Norum, K. R. Personal communication.

of red cells consequent to their loss of membrane cholesterol would presumably enhance splenic entrapment, whereas the addition of surface to a cell already sphered would help it to escape initial splenic trapping. Evidence strongly supporting these speculations has recently been obtained in studies of hereditary spherocytosis red cells which became osmotically normal and evidenced greatly improved survival after transfusion into patients with obstructive jaundice (50).

Equilibration of radioactive cholesterol between red cells and serum occurs in 4–8 hr in vitro (55–56) and in vivo (43–45). In contrast, phospholipid exchange with serum occurs more slowly, the rate of phosphatidyl choline turnover having been estimated at 7% in 12 hr (16). The speed of change in red cell surface and cholesterol content in the present study is consistent with the mobility of the red cell cholesterol compartment. Increments or decrements in phospholipid content which might occur over a much longer time course cannot be excluded by these studies in vitro which span only 24 hr. An increase in phospholipid, predominantly lecithin, has been found in the red cells of some patients with chronic liver disease (40). It is unknown whether the gain in phospholipid observed in such patients reflects a process analogous to that described here for cholesterol.

The ratio of change in the red cell surface area to the change in the red cell cholesterol in this study is approximately 1:3. This is consistent with the calculations which suggest that cholesterol contributes 30% of the total surface area of red cell lipids (57–59). Moreover, this implies that red cell membrane protein, which accounts for about 60% of the red cell ghost by weight (14), does not form a matrix which determines surface area, a fact also suggested by Maddy's inability to find red cell membrane protein in the β-configuration (57). These studies do not shed further light on Murphy's suggestion (18) that cholesterol may not be evenly distributed throughout the red cell membrane but rather is concentrated at the areas of greatest convexity. They do indicate, however, that the structure and permeability character of the red cell membrane do not depend upon a fixed unit of ratio of lipid moieties, as implied by some theories of membrane structure. Furthermore, they indicate that red cell size and shape can be modified in vivo by altering the cholesterol composition without adversely affecting membrane function or integrity.

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