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ERYTHROCYTE LIPID COMPOSITION AND SODIUM TRANSPORT IN HUMAN LIVER DISEASE

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Summary

In patients with liver disease there are usually increases in erythrocyte cholesterol and phosphatidylcholine concentrations. This increase in membrane lipid changes the shape of the erythrocyte and "spur" or "target" cells may be present. Sodium fluxes were measured in erythrocytes from 17 patients with a variety of liver diseases and from 17 normal subjects and the values related to the lipid content of the membrane. Ouabain-insensitive and ouabain-sensitive effluxes were lower in patients than in normal subjects and the reduction in ouabain-insensitive efflux was more marked. Sodium influx was also significantly lower in erythrocytes from patients than controls. Ouabain-sensitive and ouabain-insensitive effluxes and sodium influx did not correlate with the cholesterol content of erythrocytes from patients. Significant negative correlations were noted between ouabain-insensitive sodium efflux ($r = -0.63$, $P < 0.01$), sodium influx ($r = -0.61$, $P < 0.01$) and intracellular sodium concentration ($r = -0.66$, $P < 0.01$) and the cholesterol : phospholipid molar ratio of the cell but there was no significant correlation between this ratio and the ouabain-sensitive sodium efflux ($r = 0.41$, $P > 0.05$). These results support the hypothesis that an altered lipid composition may affect the permeability of the erythrocyte membrane in patients with liver disease.

Introduction

In patients with liver disease there are usually characteristic changes in the shape of the erythrocytes and "target" or "spur" cells may be present in dried blood films [1]. The abnormal morphology is accompanied by an increase in erythrocyte lipid content; spur cells have more cholesterol, and target cells more cholesterol and phospholipid [2]. In both cases the cholesterol : phospholipid molar ratio is raised. Transfusion experiments and studies in vitro have

demonstrated that both the chemical and morphological changes are reversible and result from the abnormal plasma lipoproteins found in patients with liver disease [2,3].

The cholesterol in plasma membranes appears to maintain the lipid bilayer in an intermediate fluid state by interacting with the phospholipid acyl chains [4]. Cholesterol-enriched erythrocytes, including those from patients with liver disease, exhibit a decrease in membrane fluidity when examined by a variety of physico-chemical techniques [5,6] but there have been few studies of their membrane permeability [7].

The movement of sodium out of the erythrocyte is conveniently divided into two components [8,9], that which is inhibited by cardiac glycosides such as ouabain and that which is not. Ouabain-sensitive sodium efflux represents active transport against a concentration gradient and is mediated by Mg^{2+} dependent $(Na^+ + K^+)$-ATPase (EC 3.6.1.3) [8,9]. Early reports [8,9] suggested that ouabain-insensitive sodium efflux and sodium influx had components that could be regarded as simple diffusion but there is now increasing evidence that they may be partly mediated by special transport systems [10]. In the present study we tested the hypothesis that sodium transport in erythrocytes from patients with liver disease may be affected by change in their lipid composition. Ouabain-sensitive and ouabain-insensitive sodium effluxes and sodium influx were determined in erythrocytes from patients and compared to those of normal subjects; these fluxes were then related to the lipid content of the membrane. A brief report of this work has already been made [11].

Materials and Methods

Patients

Seventeen patients with either hepatocellular or cholestatic liver disease were used in this study. All were in-patients at the Department of Medicine, Royal Free Hospital and in each case the diagnosis was established by standard clinical tests including liver biopsy. The seventeen comparison subjects were drawn from medical and laboratory staff. Venous blood was obtained from subjects after an overnight fast and anti-coagulated with heparin (10 units/ml).

Measurement of sodium fluxes

Sodium fluxes were measured by a modification of the method of Gardner et al. [12] using erythrocytes from one patient and one normal subject on the particular experimental day. The incubation medium contained NaCl 140 mmol/l, KCl 5 mmol/l, sodium phosphate buffer 3 mmol/l, $MgSO_4$ 1 mmol/l, $CaCl_2$ 2 mmol/l, glucose 10 mmol/l and albumin 100 mg/l and the magnesium chloride washing solution $MgCl_2$ 106 mmol/l, KCl 5 mmol/l with 25 ml/l of an imidazole-HCl buffer 242 mmol/l. Both solutions were prepared with distilled and doubly de-ionised water and had a final osmolality of 290 ± 5 mosM/kg water and pH 7.4.

(a) Sodium efflux. Erythrocytes were separated by centrifugation at 4°C and plasma and buffy coat removed. The cells were washed (by suspension, centrifugation and aspiration) three times in incubation medium and then suspended in an equal volume of the medium containing ^{22}Na (2 μCi/ml erythrocytes).

The suspension was pre-incubated for 3 h in a 37°C water bath with gentle inversion of the tube every 30 min and the cells then separated by centrifugation at 4°C and washed four times with ice-cold $MgCl_2$ solution. The ^{22}Na-loaded erythrocytes were added to tubes containing pre-warmed (37°C) incubation medium at a haematocrit of about 4%. The suspension was continuously rotated ("Rolamix", Luckham Ltd., Sussex, U.K.) at a constant temperature of 37°C and after 5 min two 5—6 ml aliquots poured into ice-cold tubes. One was centrifuged immediately at 4°C and 4 ml of the supernatant removed for a "zero-time" sample while 4 ml of the suspension was taken from the other tube to determine total counts. After 1 h, a further 4 ml supernatant sample was obtained from the remaining incubation suspension. Radioactivity was determined in a Wallac 1280 Ultragamma counter.

Total sodium efflux rate constant ($^eK_{Na}$) was calculated from the equation [12]:

$$^eK_{Na} = \log [1 - (\text{supernatant counts/suspension counts})]$$

The ouabain-insensitive efflux rate constant was determined by repeating the experiment with ouabain at a final concentration of 0.1 mmol/l present in the incubation medium. All efflux measurements were carried out in duplicate and the ouabain-sensitive efflux rate constant given by the difference between total and ouabain-insensitive efflux rate constants. Sodium efflux expressed as mmol/h per l erythrocyte was the product of the rate constant and intracellular sodium concentration.

(b) Sodium influx. Erythrocytes were treated in an identical manner as in efflux experiments except that ^{22}Na was not added during the 3 h pre-incubation period. After washing, some cells were used for determination of sodium and lipid concentrations whilst others were added to pre-warmed incubation medium at a haematocrit of about 4%. After a 5 min equilibration period ^{22}Na (2 μCi) was added and incubation continued for 1 h. The tubes were then centrifuged immediately at 4°C and the cells given three washes with ice-cold $MgCl_2$ solution containing ouabain (0.1 mmol/l). The cell button was haemolused with 5 ml of water and duplicate 0.15 ml aliquots removed for haemoglobin measurements followed by 4 ml for radioactivity determination. The volume of cells counted for radioactivity was calculated from the haemoglobin content of the lysate and that determined earlier for a known volume of washed cells.

Sodium influx ($^iM_{Na}$) expressed as mmol/l per h was then calculated by the following equation, which corrected for the sodium lost during the incubation period due to efflux [12]:

$$^iM_{Na} = \frac{UK}{1 - e^{-K}}$$

where U is the amount of labelled sodium taken up by the erythrocytes (calculated from the radioactivity in the cells and the specific activity of the incubation medium) and K the efflux rate constant.

Erythrocyte sodium and lipid concentrations

Washed erythrocytes obtained after the 3 h preincubation period were used

for these estimations. One portion of the cells was haemolysed and aliquots were used for estimation of haemoglobin after its conversion to cyanmethaemoglobin and for determination of sodium concentration with an Instrumentation Laboratory 343 flame photometer. A second aliquot was taken for haematocrit determination whilst the lipids were extracted [13] from a third portion. Aliquots of the lipid extract were taken for cholesterol [14] and phospholipid [15] estimations and were carried out in duplicate. Erythrocyte phospholipids were separated on silica gel H (Merck) by two-dimensional thin-layer chromatography with chloroform/methanol/aqueous ammonia (65 : 35 : 5, by vol.) as the first solvent and chloroform/acetone/methanol/acetic acid/water (50 : 20 : 10 : 10 : 5, by vol.) as the second [16]. In preliminary experiments individual fractions were identified by comparison with authentic phospholipid standards (Sigma Chemical Co., St. Louis, Mo.) and by use of specific chemical spray techniques [16]. The fractions were located with iodine vapour, scraped from the plate and the inorganic phosphorus measured after digestion with H_2SO_4 [15]. Recovery of lipid phosphorus was in the range 95—98% for both patients and normal subjects.

Statistics

All results are expressed as means ± S.D.; statistical differences were determined by Students' *t* test.

Results

Erythrocyte lipids

A comparison of erythrocyte lipid composition between patients and normal subjects is shown in Table I. Cholesterol concentration was increased ($P < 0.001$) in erythrocytes from patients; mean phospholipid content was not significantly greater although two patients, both showing "target" cells in their dried blood films, had values higher than the normal range. The mean cholesterol : phospholipid molar ratio was raised in patient erythrocytes (1.03 ± 0.14 compared to 0.86 ± 0.03 in the normal group, $P < 0.001$). The composition of the phospholipids was also changed in patient erythrocytes with an increase in the phosphatidylcholine proportion and decreases in sphingomyelin and the major acidic phospholipids, phosphatidylethanolamine and phosphatidylserine (Table I). These results were therefore in agreement with those of other workers [1,2]. Only one patient had a relative concentration of phosphatidylcholine within the normal range.

Sodium fluxes

The ouabain-insensitive sodium efflux rate constant was lower ($P < 0.01$) in erythrocytes from patients whilst the ouabain-sensitive efflux rate constant was unchanged (Table II). The sodium influx rate constant for patient erythrocytes was also decreased ($P < 0.05$) when compared to those from normal subjects. Erythrocyte sodium concentrations after pre-incubation in the standard incubation medium were higher ($P < 0.05$) for the normal subjects (6.9 ± 0.9 mmol/l) than for the patients (6.0 ± 1.3 mmol/l). Sodium influx and both ouabain-sensitive and ouabain-insensitive effluxes were all significantly reduced in ery-

TABLE I

LIPID COMPOSITION OF ERYTHROCYTES FROM PATIENTS WITH LIVER DISEASE AND FROM NORMAL SUBJECTS

Results are expressed as means ± S.D. for the number of subjects given in parenthesis. Significant differences from normal values are indicated in the footnotes. LP, lysophosphatidylcholine; SM, sphingomyelin; PI, phosphatidylinositol; PS, phosphatidylserine; P, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid.

Subjects	Cholesterol (mol/ml)	Phospholipid (mol/ml)	Cholesterol/ phospholipid (mol/mol)	% Total lipid phosphorus (mol%)						
				LP	SM	PI	PS	P	PE	PA
Normal (17)	3.30 ±0.23	3.86 ±0.25	0.86 ±0.03	1.2 ±0.3	26.7 ±1.4	0.5 ±0.3	12.7 ±0.8	29.9 ±1.1	27.2 ±1.3	1.9 ±0.5
Patients (17)	4.12 *** ±0.72	3.96 ±0.48	1.03 *** ±0.14	0.9 * ±0.5	23.8 ** ±3.8	0.7 ±0.4	11.6 * ±1.8	40.2 *** ±8.2	21.1 *** ±5.3	1.9 ±0.5

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$.

TABLE II

SODIUM EFFLUX AND INFLUX RATE CONSTANTS IN ERYTHROCYTES FROM PATIENTS WITH LIVER DISEASE AND FROM NORMAL SUBJECTS

Results are expressed as means ± S.D. for the number of subjects given in parenthesis. Significant differences from normal are indicated in the footnote. Na_i, intracellular sodium; $^eK_{Na}$, sodium efflux rate constant; $^iK_{Na}$, sodium influx rate constant.

Subjects	Na_i	$^eK_{Na}$			$^iK_{Na}$
	(mmol/l erythrocytes)	Total	Ouabain-sensitive	Ouabain-insensitive	
Normal (17)	6.9 ± 0.9	0.334 ± 0.056	0.236 ± 0.048	0.099 ± 0.024	0.0154 ± 0.0019
Patients (17)	6.0 ± 1.3 *	0.309 ± 0.063	0.238 ± 0.053	0.071 ± 0.019 **	0.0137 ± 0.0019 *

* $P < 0.05$.
** $P < 0.001$.

throcytes from patients when expressed as mmol/h per l cells. The decrease was most marked for the ouabain-insensitive sodium efflux (0.42 ± 0.13 mmol/l per h compared to 0.67 ± 0.17 mmol/l per h for the normal subjects).

Relation of sodium fluxes to erythrocyte lipid content

The cholesterol content of erythrocytes from patients did not correlate with ouabain-sensitive or ouabain-insensitive sodium efflux or with sodium influx (Table III). However, significant negative correlations ($P < 0.01$) were noted between the cholesterol : phospholipid molar ratio and ouabain-insensitive sodium efflux, sodium influx and intracellular sodium concentration. Ouabain-sensitive sodium efflux did not correlate with the cholesterol : phospholipid ratio. No correlations were seen between the sodium fluxes and the relative or absolute amounts of phosphatidylcholine or phosphatidylserine. In contrast to these results the cholesterol content of erythrocytes from normal subjects (data not shown in the table) correlated positively with ouabain-insensitive sodium efflux ($r = +0.65$, $P < 0.01$) and sodium influx ($r = +0.52$, $P < 0.05$) although no correlation was noted for ouabain-sensitive sodium efflux ($r = +0.35$, $P > 0.05$).

TABLE III

CORRELATION COEFFICIENTS BETWEEN ERYTHROCYTE LIPIDS AND SODIUM FLUXES IN PATIENTS WITH LIVER DISEASE

N.S., not significant; C : P, cholesterol : phospholipid molar ratio; $^eM_{Na}$, sodium efflux (mmol/l per h); $^iM_{Na}$, sodium influx (mmol/l per h); Na_i, intracellular sodium (mmol/l).

Variables	Correlation coefficient	Significance
Cholesterol, $^eM_{Na}$ (ouabain-sensitive)	−0.30	N.S.
Cholesterol, $^eM_{Na}$ (ouabain-insensitive)	−0.45	N.S.
Cholesterol, $^iM_{Na}$	−0.38	N.S.
C : P, $^eM_{Na}$ (ouabain-sensitive)	−0.41	N.S.
C : P, $^eM_{Na}$ (ouabain-insensitive)	−0.63	$P < 0.01$
C : P, $^iM_{Na}$	−0.61	$P < 0.01$
C : P, Na_i	−0.66	$P < 0.01$

Discussion

The results of the present study show that sodium transport in erythrocytes from patients with liver disease differs from that in erythrocytes from normal subjects. These differences appear to be related to changes in membrane lipid composition.

Ouabain-sensitive efflux of sodium from the cell occurs against a concentration gradient by action of the membrane-bound enzyme ($Na^+ + K^+$)-ATPase [8,9] which requires phospholipid (probably phosphatidylserine) for activity [17,18]. Chemical and immuno-chemical techniques have shown the enzyme to span the membrane [19] and several recent studies have emphasised the importance of membrane fluidity in determining its activity [18,20,21]. The decreased fluidity of cholesterol-enriched cells [5,6] would be expected to inhibit ($Na^+ + K^+$)-ATPase activity and support for this concept was obtained by Kroes and Ostwald [22] who reported a 60% decrease in ouabain-sensitive sodium efflux from guinea pig erythrocytes enriched with cholesterol by dietary means. However, the present study suggests that the increased cholesterol content of patient erythrocytes does not have a major effect on the activity of the sodium pump. A similar observation was made by Cooper et al. [23] who were unable to find changes in ouabain-sensitive sodium efflux when human erythrocytes were enriched with cholesterol. In contrast, addition of cholesterol to the phospholipid environment of isolated ($Na^+ + K^+$)-ATPase caused marked inhibition of its activity [20,24]. This led to the suggestion that in intact membranes cholesterol is excluded from the immediate vicinity of the enzyme [20,24,25]. Some evidence exists to support this [26] but the explanation has been questioned by Giraud et al. [27], who showed that cholesterol may have two effects on the sodium pump. One is the inhibition of pump turnover leading to a decrease in ouabain-sensitive sodium efflux, while the second is to increase the affinity of the pump for internal sodium, thereby increasing its efflux. Clearly a more detailed knowledge of the arrangement of cholesterol in the membrane bilayer when it is in excess of phospholipid [20] and the importance of clustering or lateral-phase separation of phospholipid [28,29] in relation to the membrane-bound enzyme are relevant to a greater understanding of these conflicting observations.

The decreases in ouabain-insensitive sodium efflux and sodium influx in erythrocytes from patients may be related to tighter packing of the lipid bilayer and its decreased fluidity since several studies have shown correlations between the cholesterol : phospholipid ratio and membrane fluidity [30]. The reason for the positive correlation between ouabain-insensitive sodium efflux or sodium influx and the cholesterol content of erythrocytes form normal subjects is less clear. The suggestion that cholesterol can cause phase separation of proteins [31,32], as well as decreasing membrane fluidity may be relevant since this may be related to an increase in ion permeability [33]. A decrease in ouabain-insensitive sodium efflux was observed in cholesterol-rich erythrocytes from guinea pigs [22] although Cooper et al. [23] did not find major changes in sodium influx in cholesterol-rich human erythrocytes. In both these studies the phospholipid composition of the cells was unchanged. Direct comparison with the present study is not strictly possible since there is evidence that phos-

pholipid changes may alter erythrocyte properties [34,35]. However, it seems probable that the modulation of erythrocyte membrane fluidity in liver disease by increased phosphatidylcholine content or altered phospholipid fatty acid composition [36] is only minor compared to that of cholesterol, since Vanderkooi et al. [5] showed that lipid bilayer fluidity in cholesterol-rich cells was essentially the same as that in cells from patients with liver disease, provided that both had the same cholesterol : phospholipid ratio. Differences between the results of the present experiments and those of Cooper et al. [23] are therefore more likely to be related to the smaller number of observations in their study than to differences in phospholipid composition of the erythrocytes.

The erythrocyte membrane is considered to be a satisfactory model for the lipid bilayer of plasma membranes [37]. The results presented in this paper indicate that the altered lipid composition of the erythrocyte in liver disease can affect the permeability of the membrane and its ability to transport sodium. No further attempt was made to characterize the defect although it would be of interest to know whether the furosemide-sensitive co-transport system [38] or the sodium/sodium exchange system [10] are affected. Nevertheless, the reason for the defect appears to be related to decrease in membrane fluidity as indicated by an increase in the cell cholesterol : phospholipid ratio. A recent review by Cooper [30] has stressed the importance of abnormality of cell-membrane fluidity in the pathogenesis of disease. The increased concentrations of erythrocyte cholesterol and phosphatidylcholine in liver disease are acquired from the abnormal lipoproteins present in the plasma [2,3]. The possibility that the plasma membrane lipid composition of other cells will also be altered merits further attention as this may lead to a better understanding of the cellular disturbance and metabolic abnormalities seen in liver disease.

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