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Modulation of human erythrocyte shape and fatty acids by diet

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A semi-synthetic diet (Vivonex) was administered via nasogastric tube to three cystic fibrosis patients with pancreatic exocrine deficiency for 14 days to gain weight. Dietary essential fatty acids were provided as safflower oil, which constituted 1.3% of total calories. Plasma and red blood cells were analyzed for the content and composition of lipids at the start of the diet and at days 7 and 14 of the dietary period, and the results were correlated with the morphology of the cells. Feeding Vivonex to the patients led to an essential fatty acid deficiency, which was manifested in a 50% decrease in the linoleic acid content of the phosphatidylcholine of plasma and red blood cells at days 7 and 14 and in a 20% decrease in the linoleic acid content of red cell phosphatidylethanolamine at day 14. There was no significant alteration in the levels or composition of the other phospholipid classes and in the free cholesterol/phospholipid ratio. The decrease in the linoleic acid content of the erythrocytes was accompanied by a dramatic increase in the proportion of cells as echinocytes. We conclude that restricted linoleic acid availability in cystic fibrosis patients causes a change in red blood cell shape either directly by decreasing the linoleoylphosphatidylcholine content of the membrane or indirectly by affecting enzyme activity.

Introduction

Previous work in rats has shown that a diet low in linoleic acid leads to a reduction in the proportion of linoleic acid in red cell phosphatidylcholine and to a decrease in the proportion of red cell discocytes with an increase in echinocytes [1]. Likewise, the proportion of discocytes decreased and that of spirocytes and echinocytes increased when phosphatidylcholine donor molecules low in linoleic acid were incubated with rat erythrocytes [2].

Cystic fibrosis patients with pancreatic exocrine insufficiency, while receiving a regular diet and pancreatic enzyme supplements, have a reduced linoleic acid content in red blood cell phosphatidylcholine [3]. It would be anticipated that cystic fibrosis patients with pancreatic insufficiency and receiving a semi-synthetic diet with all its linoleic acid in the form of triacylglycerol will show a further reduction in red cell linoleic acid in the absence of exogenous pancreatic enzyme supplementation. The latter dietary practice has been widely reported [4–7]. The effect of a phosphatidylcholine low in linoleic acid content on human red cell morphology has not been determined in vivo or in vitro. The opportunity to investigate the effects of a semi-synthetic diet, containing all its linoleic acid in the form of triacylglycerol, on red cell lipid content and morphology arose during short-term nutritional rehabilitation of several underweight patients with cystic fibrosis and pancreatic insufficiency. The semi-synthetic diet was

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administered as the sole source of food for a period of 14 days in the absence of supplementation with pancreatic enzymes. The present report describes the effect on red cell lipid content and morphology.

Materials and Methods

Subjects

The study group consisted of three cystic fibrosis patients (aged 16–18 years) with pancreatic exocrine deficiency. The presence of cystic fibrosis and pancreatic deficiency in each subject was established by abnormal sweat chlorides and steatorrhea. Their weight and triceps, biceps, subscapular, and suprailiac skinfold measurements were done by standard techniques [8]. Body fat was determined from the measurement of skinfold thickness [9]. A 25-year-old female in good health and with no known disease and subsisting on a normal North American diet provided control red blood cells and plasma.

Diet

The composition of the semi-synthetic diet (Vivonex, Eaton Laboratories, Norwich, NY) as percent kilocalories of carbohydrate, protein and fat was 90.2, 8.5 and 1.3, respectively. The fat source, safflower oil, provided 1% of total calories as linoleic acid. The Vivonex was administered by a continuous nasogastric drip for 14 days; no pancreatic enzymes or other nutrient supplementation were provided.

Isolation of cells

Blood was drawn in the presence of EDTA and the cells were separated from plasma by centrifugation at $200 \times g$ for 20 min in a refrigerated centrifuge. The cells for lipid analysis were washed three times in 154 mM NaCl to remove residual plasma and the buffy coat.

Total lipid extracts of the red blood cells were prepared as described by Rose and Oklander [10]. The phospholipid classes were resolved by one-dimensional thin-layer chromatography using chloroform/ methanol/ acetic acid/ water (100 : 45 : 20 : 6, v/v) as the developing solvent [11]. The phosphorus content of each band of the total lipid extract was measured by the method of Bartlett [12] following lipid extraction. The fatty acid composition of the individual phospholipid classes was determined by gas-liquid chromatography following transmethylation with boron trifluoride/ methanol [13]. The gas-liquid chromatography of the fatty acid methyl esters was performed on a Hewlett-Packard model 5840 gas chromatograph equipped with a flame ionization detector and a 2 m × 0.3 cm i.d. glass column packed with 3% Silar 10C on 100–120 mesh Gas Chrom W (Applied Science Laboratories, State College, PA). The column oven was programmed from 150–200°C at 5 Cdeg per min. The injector temperature was 250°C, as was the detector temperature. The carrier gas was nitrogen at 40 ml/min. The fatty acid methyl esters were identified by reference to a standard mixture of fatty acid methyl esters of known composition. Peak areas were computed by electronic integration and the fatty acid concentrations were expressed on a weight percent basis. Free cholesterol was determined by GLC [14].

Statistical analysis

The analytical results were compared at 0, 7 and 14 days using the one-way analysis of variance. Duncan's standard range test was used to determine the difference between control and patient measurements as well as time differences between patients.

Electron microscopy

Control and patient fresh red cells were fixed in 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), sedimented on glass slides, dehydrated in a graded series of alcohol and dried (critical point). After a coating with gold, they were examined using a JEOL 35 Autoscan scanning electron microscope. Cells were classified as discocytes, echinocytes I, echinocytes II, echinocytes III, or unknowns, by an independent observer, a trained hematologist with no prior knowledge of patient or control subject diagnosis. Discocytes are characterized by smooth biconcave structures, echinocytes I as irregularly contoured discocytes, echinocytes II as flat red cells with spicules, and echinocytes III as spherical cells with spicules. The morphology of the cells is presented for each type of cell observed in 100 randomly counted cells

using the electron micrographs of the patients and of the control subject.

Results

The means and standard deviations for age, energy consumed and weight gain were respectively 18.8 ± 2.7 years, 3275 ± 770 kcal/day and 2.6 ± 0.5 kg. 80% (2.1 ± 0.4 kg) of the weight gain resulted from increased body fat.

Fig. 1 compares the electron micrographs obtained for the red blood cells of the control subject and for a representative patient at 0, 7 and 14 days of the dietary experiment. There were obvi-

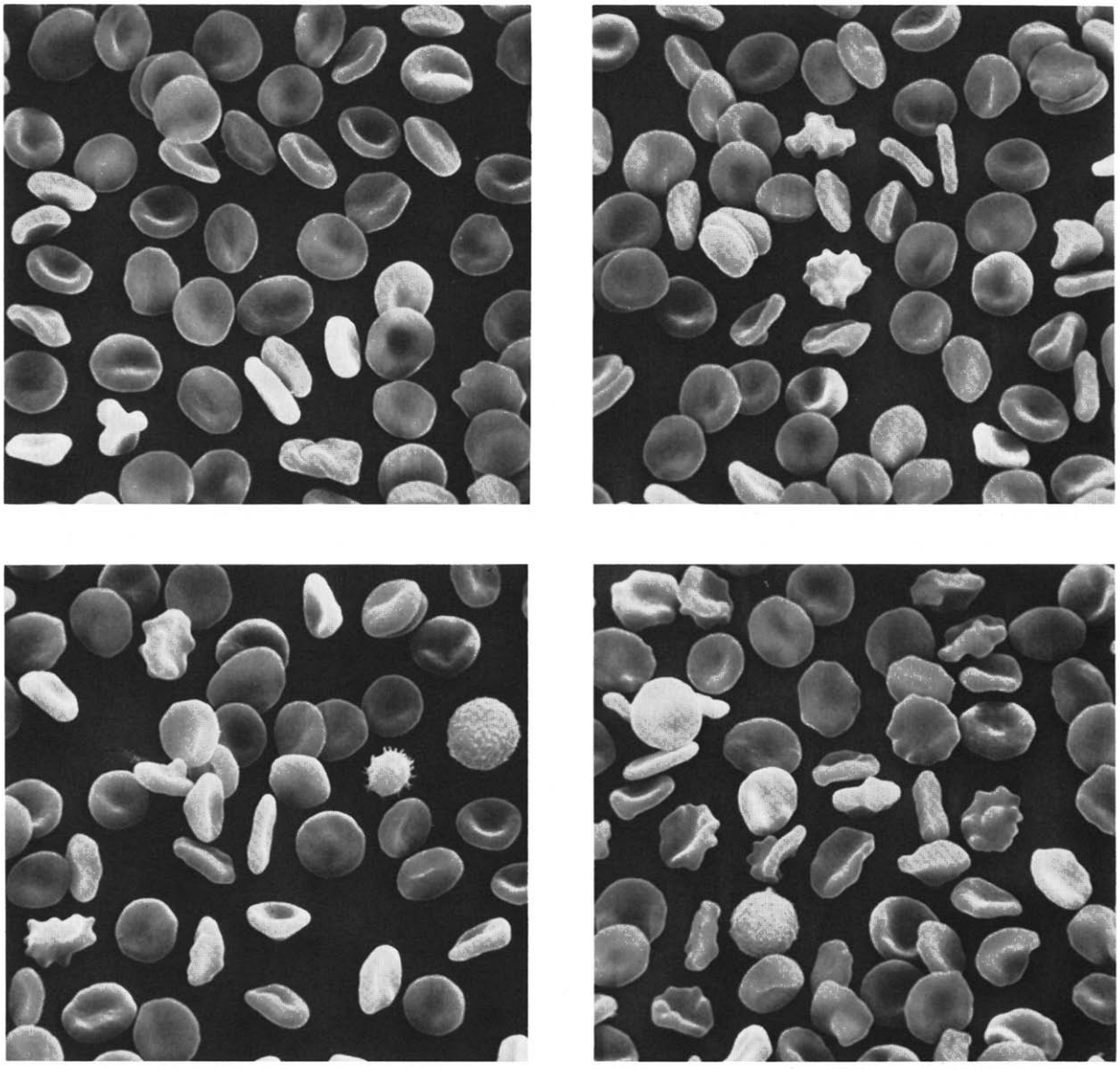

Fig 1. Scanning electron microscopic examination of human erythrocytes. Top left, erythrocytes from control subject on a regular North American diet; top right, bottom left and right, erythrocytes from cystic fibrosis patients at 0, 7 and 14 days of continuous enteral Vivonex therapy. The normal subject shows minimal echinocytes, while the cystic fibrosis patients show increased echinocytosis with duration of therapy (see Table I for quantitation).

TABLE I

RED BLOOD CELL MORPHOLOGY IN CONTROL AND CYSTIC FIBROSIS (CF) PATIENTS AT 0, 7 AND 14 DAYS OF THERAPY (MEAN ± S.E.)

	Control	CF subjects		
		0 days	7 days	14 days
Discoid	83.33 ± 4.18	57.67 ± 3.44 [a]	35.00 ± 5.20 [a,b]	28.33 ± 8.70 [a,c]
Echinocytes I	15.00 ± 4.51	31.67 ± 1.45	49.69 ± 5.34 [a]	51.33 ± 8.70 [a]
Echinocytes II	0.33 ± 0.33	6.33 ± 4.85	9.00 ± 4.17	16.33 ± 2.85 [a]
Echinocytes III	0	1.33 ± 1.33	0	0.67 ± 0.67
Unknown	1.00 ± 0.58	3.00 ± 1.73	6.33 ± 2.34	3.33 ± 2.41

[a] $P < 0.05$ (control vs. CF 0, 7 and 14 days).
[b] $P < 0.05$ (CF 0 vs. CF 7 days).
[c] $P < 0.05$ (CF 0 vs. CF 14 days).

ous differences in the proportions of the various cell types between the normal subject and the patient at the beginning of the study, which were greatly increased upon progressing dietary treatment. The results are quantitatively evaluated for the control and patients in Table I. Thus, while the control subject contains about 83% discocytes and 15% echinocytes I with no detectable echinocytes II or III, the patients had only 58% discocytes and 32% echinocytes I and 6% echinocytes II. After 7 days of Vivonex feeding the discocyte proportion was reduced to 35% with an increase in echinocytes I to 50%. These changes were found to persist on day 14, when the proportion of echinocytes II had increased to 16%.

The thin-layer patterns obtained for the different phospholipid classes of the red cells of one of the patients at days 0, 7 and 14 of the experiment showed no discernible discrepancies in the qualitative or quantitative distribution of the bands. The results of the phosphorus analysis of the bands are given in Table II. The diet had no effect on the phospholipid class composition of the red cells.

Table II shows that the diet also had no effect on the free cholesterol/total phospholipid ratio of the red blood cells, which remained close to one, also found for normal subjects [15]. Likewise, within our patients, there was no change in the red blood cell fatty acid composition of the phosphatidylethanolamine between day 0 and day 7

TABLE II

CHOLESTEROL/PHOSPHOLIPID RATIO AND PHOSPHOLIPID COMPOSITION OF ERYTHROCYTE MEMBRANE OF CYSTIC FIBROSIS PATIENTS AT 0, 7 AND 14 DAYS OF THERAPY

Values are shown as means ± S.E. of percent of phosphorus. C/PL = cholesterol/phospholipid ratio.

Phospholipids	Composition (%)		
	0 days	7 days	14 days
Phosphatidic acid/phosphatidylglycerol	2.83 ± 0.95	3.40 ± 0.88	3.80 ± 1.74
Phosphatidylethanolamine	30.15 ± 5.21	30.85 ± 8.17	30.25 ± 3.40
Phosphatidylserine	11.59 ± 1.26	12.63 ± 0.67	10.79 ± 2.50
Phosphatidylinositol	2.07 ± 1.41	2.86 ± 1.58	1.21 ± 0.84
Phosphatidylcholine	25.69 ± 4.00	25.61 ± 2.87	27.81 ± 2.37
Sphingomyelin	25.89 ± 1.73	23.51 ± 4.01	23.82 ± 6.56
Lysophosphatidylcholine	1.87 ± 0.87	1.45 ± 1.45	2.32 ± 1.92
C/PL (mol/mol)	0.93 ± 0.03	0.95 ± 0.07	0.90 ± 0.06

TABLE III

FATTY ACID COMPOSITION (MEAN % ± S.E.) OF RBC PHOSPHATIDYLETHANOLAMINE OF CONTROL AND CYSTIC FIBROSIS PATIENTS AT 0, 7 AND 14 DAYS OF THERAPY

Fatty acid	Control	CF subjects		
		0 days	7 days	14 days
16:0	19.43 ± 0.41	18.80 ± 0.65	22.80 ± 2.14	20.67 ± 1.42
18:0	10.57 ± 0.84	9.70 ± 0.95	13.35 ± 3.03	9.53 ± 0.55
18:1($n-9$)	22.20 ± 0.61	21.27 ± 0.35	24.93 ± 3.94	20.83 ± 1.10
18:2($n-6$)	5.50 ± 0.15	5.77 ± 0.43	4.77 ± 0.57	4.17 ± 0.12 [a]
20:4($n-6$)	28.70 ± 1.15	30.20 ± 1.57	23.97 ± 7.55	30.10 ± 1.44
22:5 and 22:6($n-3$)	11.23 ± 0.82	10.20 ± 1.24	10.27 ± 0.97	11.03 ± 1.76

[a] $P < 0.05$ CF, 0–14 days.

TABLE IV

FATTY ACID COMPOSITION (MEAN % ± S.E.) OF RBC PHOSPHATIDYLCHOLINE OF CONTROL AND CYSTIC FIBROSIS PATIENTS AT 0, 7 AND 14 DAYS OF THERAPY

$P < 0.05$, [a] control–CF; [b] CF, 0–7 days; [c] CF, 0–14 days.

Fatty acid	Control	CF subjects		
		0 days	7 days	14 days
16:0	30.67 ± 1.10	31.67 ± 3.13	37.15 ± 4.18	34.83 ± 2.14
16:1	3.40 ± 1.64	4.07 ± 0.32	3.67 ± 0.40	9.03 ± 1.55 [a]
18:0	10.20 ± 0.28	12.33 ± 1.69	12.13 ± 3.11	12.00 ± 1.56
18:1	19.67 ± 0.62	25.07 ± 0.80 [a]	23.30 ± 3.28	27.67 ± 2.20
18:2	21.30 ± 1.80	15.63 ± 0.78 [a]	8.63 ± 1.91 [a,b]	8.53 ± 1.03 [a,c]
20:3($n-9$)	–	–	0.30 ± 0.17	1.83 ± 0.73
20:4	9.47 ± 1.01	6.63 ± 1.98	5.23 ± 1.60	6.33 ± 0.73

TABLE V

FATTY ACID COMPOSITION (MEAN ± S.E.) OF SERUM PHOSPHATIDYLCHOLINE OF CONTROL AND CYSTIC FIBROSIS PATIENTS AT 0, 7 AND 14 DAYS OF VIVONEX THERAPY

$P < 0.05$, [a] controls–CF; [b] CF, 0–7 days; [c] CF, 0–14 days; [d] CF, 7–14 days.

Fatty acid	Control	CF subjects		
		0 days	7 days	14 days
16:0	29.10 ± 1.08	28.33 ± 1.80	27.60 ± 1.74	27.97 ± 1.49
16:1	1.60 ± 0.50	3.03 ± 0.22 [a]	4.50 ± 0.38 [a,b]	4.90 ± 0.36 [a,c]
18:0	11.70 ± 0.27	10.20 ± 0.60	12.50 ± 0.82 [a]	10.73 ± 0.79 [d]
18:1	16.50 ± 0.98	18.07 ± 1.48	25.73 ± 2.0 [a,b]	25.60 ± 3.10
18:2	21.00 ± 0.74	16.07 ± 1.70 [a]	7.30 ± 1.50 [a,b]	6.57 ± 0.43 [a,c]
20:3($n-9$)	–	0.82 ± 0.40	1.73 ± 0.35	2.63 ± 1.22
20:4	12.50 ± 0.05	11.03 ± 1.08	9.43 ± 1.65	7.80 ± 0.60 [a]

(Table III) or of sphingomyelin (data not shown). By day 14, patients compared to control, the linoleic acid content of phosphatidylethanolamine was significantly decreased in comparison to day 0 ($P < 0.05$) (Table III). The morphological changes in the red blood cells were accompanied by a marked decrease in the linoleic and an increase in the oleic acid content of the phosphatidylcholine of the red blood cells. Table IV shows that, over a 7-day period, the linoleic acid content decreased from about 16 to 9%. This decrease persisted over the next 7 days, when an increase in palmitoleic acid (4 to 9%) and in the eicosatrienoic acid (0.3 to 1.8%) was also observed. The alterations in the fatty acids of the phosphatidylcholine of the red blood cells caused by the diet paralleled those seen in the fatty acids of the serum phosphatidylcholine (Table V).

Discussion

The major sequence of events in our patients can be postulated as follows: (1) an inadequate dietary intake or endogenous release of essential fatty acids (linoleic acid); (2) increased formation of plasma phosphatidylcholine containing monosaturated species; (3) exchange of the plasma phosphatidylcholine with red cell phosphatidylcholine; and (4) altered species of membrane phospholipid affecting membrane morphology. This sequence occurred rapidly, for the morphological changes were already present after 1 week and therefore could not be due to newly synthesized red cells formed with the altered phosphatidylcholine species.

To prevent an aggravation of the essential fatty acid deficiency in our patients, the diet had to contain at least 1% of total energy as essential fatty acids in an absorbable form [16]. Vivonex contains 1% of total calories as essential fatty acid (linoleic acid). Failure to add supplemental pancreatic enzyme to the dietary intake of our patients apparently deprived them of the opportunity to hydrolyze the linoleic acid and to absorb it along with the carbohydrate nutrients [7]. In addition, the continuous administration of carbohydrates above caloric requirements prevented the mobilization of the large linoleic acid stores in adipose tissue [17]. In fact, the high energy intake favoured lipogenesis as the body fat increased by 2.1 ± 0.4 kg during the 14 days of therapy.

The fatty acid composition of the phosphatidylcholine of plasma and red cells of the normal subject and of the cystic fibrosis patients at the start of the study corresponded closely to previously reported values [3,18]. Also, in agreement with Rogiers et al. [3], our study showed that the change in the red cell linoleate content was confined essentially to phosphatidylcholine. The parallel changes in the fatty acid composition of plasma and red cell phosphatidylcholine support the previously described renewal of red cell phosphatidylcholine by exchange with plasma [19].

Turnover of the mature human erythrocyte phospholipid fatty acids in situ is limited to deacylation of endogenous phospholipid and reacylation of the resulting lysophospholipid [20–22] and/or exchange of intact phospholipid molecules with exogenous phospholipid because these cells cannot alter fatty acid chain length or degree of unsaturation or synthesize phospholipid de novo [23]. In this respect the second process involves a transfer of phospholipids from the serum lipoproteins to the red cell membrane and vice versa and appears to be the dominant pathway for renewal of phosphatidylcholine in mature red blood cells [24].

Studies with molecular probes [25,26], nonpenetrating enzymes [27–29] and exchange proteins [2,30] provide evidence that approx. 70% of phosphatidylcholine, 80% of sphingomyelin and 20% of phosphatidylethanolamine are located in the outer layer of the human red blood cell, whereas the remaining phosphatidylcholine, sphingomyelin and phosphatidylethanolamine are distributed in the inner layer. Phosphatidylserine is located exclusively in the inner layer. In this regard it has been suggested that only those phospholipids occupying the outer half of the human red cell lipid bilayer are exchangeable [24,31]. It has been estimated that about 75% of the phosphatidylcholine and none of the phosphatidylserine are exchangeable [33]. The turnover time of the exchangeable pool of erythrocyte phosphatidylcholine has been estimated to be 5 days [24]; a finding which is supported by our results. Moreover, Child et al. [34] have demonstrated that there is preference for the unsaturated molecular

species of phosphatidylcholine in the exchange process, thus resulting in a greater loss of linoleoylphosphatidylcholine. The confinement of the fatty acid alteration in our study largely to the red cell phosphatidylcholine may be rationalized on the basis of the small exchangeable pool of phosphatidylethanolamine as well as the relative inertness of the fatty acids of sphingomyelin to dietary influence and its quantitative exchange being less than half of phosphatidylcholine [24].

The relative contributions of the direct exchange and of the acyl exchange mechanisms to the alterations observed in the fatty acid composition in the present experiments were not determined. Marinetti et al. [35] found that a very small population of phosphatidylethanolamine molecules on the outer half of the membrane had a 4-fold more rapid turnover of their fatty acids by way of acylation than the remaining phosphatidylethanolamine molecules. Moreover, this enzymatic incorporation of fatty acids into phosphatidylethanolamine showed a preference for palmitic acid over stearic, linoleic and linolenic acid. Thus differences between day 0 and day 14 of the patient phosphatidylethanolamine in linoleic acid content may have resulted from the acyl and/or exchange renewal mechanism.

The relationship between the decrease in the linoleate content of the phosphatidylcholine and the morphological distortion of the red cell membrane is unknown. Phosphatidylcholine occurs in the red cell membrane in the form of well-defined species, with the saturated fatty acids in the *sn*-1-position and the unsaturated fatty acids in the *sn*-2- position [36]. The normal red cells contain 5% disaturated species. The loss of discocytes and the concomitant increase in the number of echinocytes are associated with the level of linoleoylphosphatidylcholine in the erythrocyte. The present results are in agreement with those of Rao et al. [1], who noted that exclusion of linoleic acid from the diet led to a reduction of linoleic acid in the phosphatidylcholine of red blood cells and an increase in the proportion of echinocytes of rats within 3–7 days. The latter study also showed that the loss of discoid shape was reversed within 7 days by a diet rich in linoleic acid. Likewise, Lange et al. [2] showed, in vitro, that when monosaturated or saturated molecular species of phosphatidylcholine replaced 25% of the native red cell phosphatidylcholine species, echinocyte formation and hemolysis occurred. When native red blood cell phosphatidylcholine was replaced by egg phosphatidylcholine no morphological abnormalities occurred. On the basis of the present results, it may be calculated that about 15% ($2 \times 7.5\%$ decrease in 18 : 2) of the total phosphatidylcholine was replaced by monosaturated phosphatidylcholine, which may have been responsible for the observed increase in the proportion of the echinocytes in the red blood cells of the patients on the Vivonex therapy.

Other modifications of the lipid content of erythrocyte membrane are known to affect red cell morphology, e.g., partial depletion of cholesterol from the erythrocyte [37]; increased content of erythrocyte lysophosphatidylcholine [38]; and accumulation of phosphatidylcholine containing short-chain fatty acids [39]. Clearly, the diet had no affect on the concentrations of red cell cholesterol and phospholipid classes and cannot provide an explanation for the observed morphological alterations. Thus, alteration of one of the major red cell membrane constituents, phosphatidylcholine, by diet, is important in relation to the maintenance of membrane integrity in vivo.

The physiological significance of these observations was not determined in this study. Internal membrane-bound enzyme functions have been shown to be altered in the red cell of cystic fibrosis patients [40,42]. Since it is well established that enzyme function can be modulated by the lipid environment [43], interpretation of alterations in the function of red cell membranes in cystic fibrosis patients should not be attempted without first assessing the lipid profiles of these membranes.

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