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Effects of essential fatty acid deficiency on various functions of the rat erythrocyte membrane*)

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With 3 figures

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Introduction

Recent clinical reports of biochemical evidence of essential fatty acid (EFA) deficiency in the course of fat-free total parenteral nutrition (3, 12, 16) have served to focus attention on the physiological aberrations associated with this deficiency state. In fact, one of the recurrent questions in this field concerns the interpretation of abnormal EFA patterns in plasma and in tissues particularly with regard to the functional consequences of these abnormalities. The likelihood that the fatty acid pattern of cell membrane lipids is altered by EFA deficiency has led to the general postulate that many of the overall physiological aberrations of EFA deficiency are secondary to changes in the structure and/or the function of the cell membrane (9). Despite the logical nature of these assumptions, functional abnormalities have not been consistently demonstrated (10) in EFA-deficient membranes. These considerations prompted the present study which was designed to explore the relationships between the biochemical changes of EFA deficiency and various functions of the rat erythrocyte membranes.

Materials and methods

Weanling male Sprague-Dawley rats were randomly assigned to either a fat-free (25) or to standard laboratory chow diet (26). Both groups were housed in metabolic cages with 4 animals per cage. Animals assigned to the fat-free diet were fed *ad libitum* and their daily intake was carefully measured. The intake of the control animals was restricted to the amount of food consumed by the fat-free group on the preceding day. Both groups received water *ad libitum*. The typical skin lesions of EFA deficiency (2) first appeared at about 50 days. Thereafter, a few animals were killed periodically to assess the biochemical severity of the deficiency, as reflected by the triene: tetraene ratio (8) of the plasma and erythrocyte lipids. After 150 days

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on the fat-free diet, the deficiency was deemed to be of sufficient severity (plasma triene/tetraene ratio > 0.40) to conduct the definitive studies of osmotic resistance, intracellular sodium and potassium concentrations and amino acid uptake in both deficient and normal erythrocytes (see below). Blood for these determinations was obtained by aortic puncture after ether anesthesia; sodium EDTA (1 mg/ml blood) was used as the anticoagulant. Lipid extract and fatty acid patterns were determined by methods previously reported (1).

Osmotic resistance

Osmotic resistance of erythrocytes from control and EFA deficient animals was determined by the method of *Hallmann* (6) using NaCl concentrations ranging from 0.70% to 0.30%. Two drops of whole blood were added to each dilution, the suspension mixed gently by inversion and centrifuged at 2,000 r.p.m. for 1 minute. Beginning hemolysis was read as the first appearance of a yellowish supernatant. Hemolysis was judged to be complete when precipitation was no longer visible.

Intracellular sodium and potassium concentrations

Aliquots of blood were washed 6 times with $MgCl_2$ (280 mOsmol) and adjusted (with $MgCl_2$) to a final packed cell volume of 30%–50% (8); 0.05 ml of this suspension was then lysed in 10 ml $LiNO_3$ (100 p.p.m.) and sodium and potassium concentrations of the lysate determined by flame photometry. All determinations were done in duplicate.

Amino acid uptake

All steps involved in studies of amino acid uptake were carried out at 15 °C, since at this temperature uptake is slowed sufficiently to permit the kinetics to be determined accurately. After washing 3 times with Tris buffer, pH 7.6 (15), an aliquot of suspended erythrocytes was incubated for 10 minutes with THO (40 μCu/ml suspension) for subsequent determination of total water content. Following this incubation, 0.5 ml of the suspension was added to equal volumes of solutions of ^{14}C-L-Leucine (with concentrations varying stepwise from 0.05 to 2 mM) and double strength buffer solution; the final osmolality of the mixture was 282 mOsm/kg H_2O. Constant mixing was achieved by use of a magnetic stirrer. After 60 seconds, the incubation was stopped by rapid centrifugation of a 0.2 ml aliquot of suspension through a 4 mm layer of silicone oil into 0.025 ml of 15% $HClO_4$ (7,14). Since the density of the oil is lower than that of the erythrocytes but higher than that of the incubation medium, only the erythrocytes passed into the acid layer where they were immediately deproteinized with the release of soluble constituents. Samples of both supernatant and lower acid layers (0.010 ml) were added to counting vials containing Bray's solution, and ^{3}H and ^{14}C were determined simultaneously using a Nuclear Chicago scintillation counter. The observed counts were corrected for background, quenching and extracellularly-trapped incubation fluid. The latter was estimated by incubating separate aliquots of washed erythrocytes with ^{14}C-Inulin or ^{14}C-Sucrose for 1, 2, and 3 minutes, centrifuging as described above, and subsequently determining the ^{14}C-content of the acid layer. On average, 23.5% of the total water in the deproteinized layer was found to be trapped extracellular fluid.

Results

Erythrocyte membrane lipids

The major fatty acids of the erythrocyte membrane lipids, expressed as percent of the total measurable peak areas of the chromatogram, are shown in Figure 1. The amounts of palmitic acid (16 : 0), palmitoleic acid

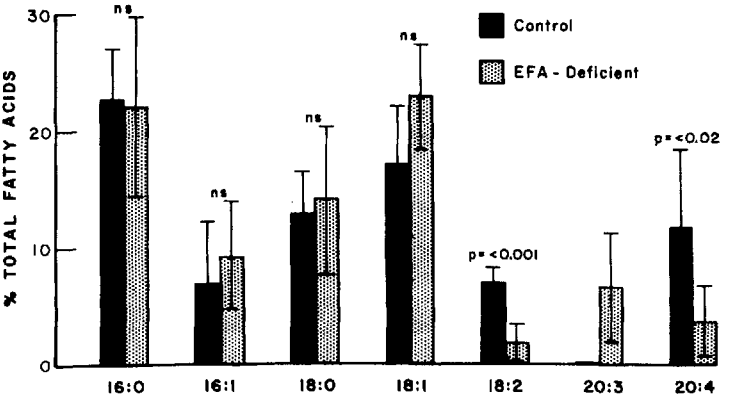

Fig. 1. Fatty acid patterns of EFA deficient and control erythrocyte lipids.

(16 : 1), stearic acid (18 : 0) and oleic acid (18 : 1) were not significantly different between the two groups, although the mono-unsaturated acids, palmitoleic and oleic, tended to be higher in the EFA-deficient membranes. On the other hand, the essential fatty acids, linoleic (18 : 2) and arachidonic (20 : 4) were significantly reduced ($p < 0.001$ and < 0.02, respectively) in the EFA-deficient membranes. Eicosatrienoic acid (20 : 3), not measurable in the membrane lipids of the control group, accounted for 6.7% of the total measurable fatty acids in the membrane lipids of the EFA-deficient group.

Osmotic resistance and intracellular cation concentrations

Table I shows a summary of the results of studies designed to determine osmotic resistance and the intracellular sodium and potassium concentrations in the EFA-deficient and control cells. No significant differences are apparent between the two groups with respect to any of these variables when the data are treated in this way, i.e., comparison of mean values.

Table 1. Osmotic resistance and intracellular Na^+ and K^+ of EFA-deficient and control groups.

Osmotic resistance	EFA-deficient	control	p
Beginning hemolysis (g NaCl/100 ml)	0.51±0.10 (8)*	0.53±0.03 (5)	n. s.
Complete hemolysis (g NaCl/100 ml)	0.37±0.01 (8)	0.40±0.03 (5)	n. s.
Intracellular [Na^+] (mEq/l ICW)**	4.28±1.36 (7)	4.75±1.49 (5)	n. s.
Intracellular [K^+] (mEq/l ICW)	107.1±10.3 (6)	103.0 ±9.5 (4)	n. s.

* ( ) indicates n in each group
** ICW indicates intracellular water

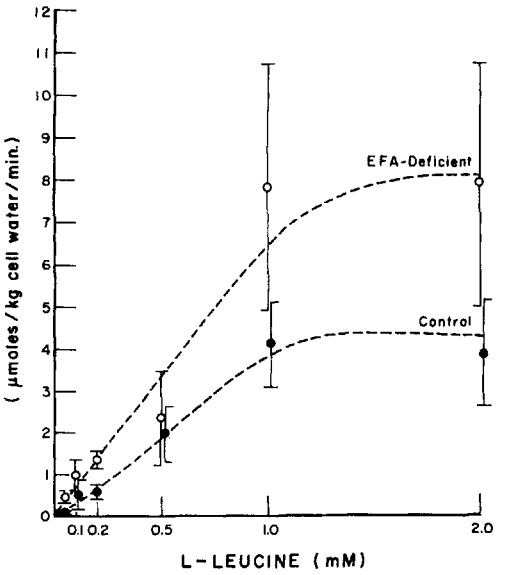

Fig. 2. Effect of extracellular leucine concentration on uptake of ^{14}C-L-leucine by EFA-deficient and normal erythrocytes.

^{14}C-L-leucine uptake

The uptake of L-leucine at 1 minute (15), as a function of stepwise increases in extracellular leucine concentrations is shown in Figure 2. Uptake by both normal and EFA-deficient cells increased as the concentration of leucine in the medium was increased from 0.05 to 1.0 mM with

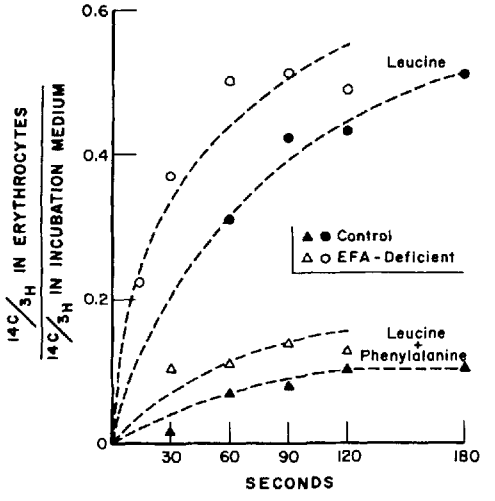

Fig. 3. Effect of L-Phenylalanine on ^{14}C-L-Leucine uptake by EFA-deficient and control erythrocytes.

no further increase in uptake being observed at higher concentrations. Statistical treatment of these uptake curves by an analysis of variance (using the log of uptake) demonstrated that the EFA-deficient erythrocytes accumulated leucine to a significantly greater extent than did the control erythrocytes ($p < 0.01$) at one minute.

In kinetic studies, ^{14}C-L-leucine uptake by deficient and control cells was determined at 30, 60, 90, 120 and 180 seconds in a medium containing leucine at a concentration of 1.0 mM, both with and without blocking levels (10 mM) of L-phenylalanine (see Figure 3). Leucine uptake in the absence of phenylalanine proceeded more rapidly and to a greater extent in the EFA-deficient cells. Phenylalanine reduced the rate and extent of leucine uptake in both types of cells, the resulting curves both being reduced by about 75 to 80% of the non-blocked values.

Discussion

With EFA deficiency, there is a characteristic fall in the essential n-6 family of fatty acids in the plasma, *viz.*, linoleic (18 : 2) and arachidonic (20 : 4), and a compensatory increase in the n-9 group, *viz.*, 5,8,11-eicosatrienoic acid (20 : 3). The results of the present study along with those published previously from this laboratory in a similar group of EFA-deficient rats (1) clearly confirm the concept that the erythrocyte membrane shows a similar pattern of changes in EFA status as observed in plasma. Since phospholipid comprises over 97% of all erythrocyte lipids (5), these changes in membrane EFA clearly involve this important fraction of the lipid phase of the membrane.

Although never conclusively demonstrated, it has been widely assumed that the changes in membranes lipids which occur with EFA deficiency are accompanied by functional derangements. Indeed, *MacMillan* demonstrated an increase in osmotic fragility of erythrocytes from rats rendered EFA-deficient by 280 days of a fat-free diet containing 2% cholesterol (11). This result is seemingly at variance with our own, although our animals were less severely deficient. Although we are unaware of clinical reports of an increased osmotic fragility in clinical EFA deficiency, to our knowledge the problem has not been systematically examined. Nor have intracellular sodium or potassium concentrations been measured as a specific function of EFA status in either an experimental or a clinical setting.

Although no statistically significant differences in the mean values for osmotic fragility or intracellular sodium and potassium concentrations between control and EFA-deficient cells were shown, there appears to be a correlation between each abnormality of erythrocyte membrane function and the degree of erythrocyte membrane lipid abnormality. For example, osmotic fragility correlated extremely well ($r = 0.92$) with the erythrocyte ratio of eicosatrienoic acid to the sum of arachidonic and linoleic acids (20:3/20:4 + 18:2). Intracellular sodium and potassium concentrations correlated equally well ($r = 0.95$ and -0.84, respectively) with the membrane triene: tetraene ratio (20:3/20:4) or the membrane ratio of eicosatrienoic acid to the sum of arachidonic and linoleic acids (20:3/20:4 + 18:2). Leucine uptake, on the other hand, showed only a moderately good correlation ($r = 0.67$) with either of the previously mentioned membrane ratios or with

the membrane ratio of eicosatrienoic acid to linoleic acid. These correlations may indicate that the degree of deficiency, as judged by both the type and degree of membrane lipid abnormality, is the crucial variable in discerning membrane abnormalities of EFA deficiency. Since the fatty acid patterns of both plasma and tissue lipids of EFA-deficient animals vary markedly from animal to animal, simply treating the mean values without regard for possible variation may be deceptive. On the other hand there is no assurance that the above correlations are biologically significant. We suggest that they are sufficiently striking to deserve further evaluation.

In contrast to the equivocal results with osmotic fragility and intracellular cation concentrations, leucine transport was clearly different between the EFA-deficient and the normal erythrocyte. We chose this particular system to study because it has been extensively studied by *Christensen* and colleagues in the Ehrlich ascites tumor cell (4). In this cell type, the leucine-preferring, or L-system of amino acid uptake, is believed to consist of a saturatable temperature-sensitive component in addition to one or more non-saturatable components (17). The former component is shared by a number of amino acids (e.g., phenylalanine, valine, methionine) and the affinity of a given amino acid for the carrier site seems to be directly proportional to the length of its hydrocarbon side chain (17).

Our results with phenylalanine, which competes with leucine for active uptake (i.e., similar inhibition in both EFA-deficient and control cells), suggest that this component of amino acid uptake is not affected by EFA deficiency. If this is the case, EFA deficiency would seem to have the effect of enhancing the passive diffusion component. This hypothesis, although not definitely proven by the present studies, is intuitively reasonable if the structural characteristics of the membrane were altered by EFA deficiency.

Finally, it should be pointed out that the many *in vivo* physiological effects of EFA deficiency, in fact, may not have as a basis the alteration of membrane structure or function. Rather, they may depend upon some other, entirely unrelated mechanism which cannot be revealed by purely *in vitro* studies. In this regard, it is noteworthy that both linoleic and arachidonic acids are precursors of prostaglandins and that this process appears to be reduced in EFA deficiency (13). Thus it is conceivable that a deficiency, excess or imbalance of particular classes of prostaglandins, substances which exert an extraordinary variety of physiological effects, underlie the physiological derangements of clinical EFA-deficiency and that this area, rather than membrane function and structure, should be explored more vigorously by students of EFA deficiency.

Summary

Various membrane transport functions have been studied in erythrocytes from essential fatty acid (EFA) deficient rats in order to determine whether or not functional abnormalities induced by documented EFA-deficiency of the membrane could be demonstrated. No differences were found between EFA deficient and control cells with respect to mean values for osmotic resistance or intracellular sodium and potassium concentrations. However, uptake of leucine by EFA deficient erythrocytes was significantly greater than that of control erythrocytes. Kine-

tic studies suggest that EFA deficiency enhances the passive diffusion component of this transport.

Zusammenfassung

An Erythrozyten von Ratten mit essentiellem Fettsäuremangel wurden verschiedene Transportfunktionen der Membran studiert, um zu klären, ob ein Mangel an essentiellen Fettsäuren in der Erythrozytenmembran zu funktionellen Veränderungen führt. Im Vergleich zu Kontrolltieren konnte bezüglich nachfolgender Parameter kein signifikanter Unterschied gefunden werden: osmotische Resistenz; intrazelluläre Natrium- und Kaliumkonzentration.

Die Aminosäure Leuzin wurde jedoch von Erythrozyten mit essentiellem Fettsäuremangel signifikant vermehrt aufgenommen als von Erythrozyten der Kontrolltiere. Kinetische Untersuchungen stützen die Vermutung, daß essentieller Fettsäuremangel die passive Komponente des Transportes verstärkt.

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