

Effect of essential fatty acid deficiency on the lipid composition of the Yoshida ascites hepatoma (AH 130) and of the liver and blood plasma from host and normal rats¹

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Abstract In order to study the response of a poorly differentiated tumor to nutritional manipulation, the Yoshida ascites hepatoma (AH 130) was grown in rats fed an essential fatty acid (EFA)-deficient diet and in rats fed a control diet. Hepatomas, livers, and blood plasma from host rats and normal rats were studied as to the effects of EFA deficiency on the lipid composition. Normal rats fed an EFA-deficient diet showed an increased concentration of triglycerides and cholesteryl esters in the liver and a reduced level of total phospholipids in plasma. Host rats fed the EFA-deficient diet showed a lower concentration of triglycerides in the liver when compared with the host rats fed a control diet. In addition, EFA-deficient host rats had reduced levels of plasma free fatty acids and triglycerides. These latter were markedly high in host rats under normal dietetic conditions. As compared to the livers of either host rats or normal rats fed the control diet, the Yoshida hepatoma cells had a lower content of total phospholipids and free fatty acids as well as a higher level of free cholesterol; they also showed a typical fatty acid pattern in their phospholipids. The main characteristics of this pattern were a high content of oleic and palmitoleic acids and a low level of C₂₀ and C₂₂ polyunsaturated fatty acids. Exposure of Yoshida hepatoma cells to an EFA-deficient environment resulted in a decrease in the concentration of total phospholipids and free fatty acids and in changes in the fatty acid composition similar to those observed in the livers of normal and host rats. These changes suggest that, under the experimental conditions used, the Yoshida hepatoma cells are responsive to EFA deficiency.

Supplementary key words ascitic plasma · argentation thin-layer chromatography · liver triglyceride · plasma triglyceride · tumor-bearing animals · oleic acid · eicosatrienoic acid · triene:tetraene ratio

It has been reported that tumor cells, as a result of the loss of some metabolic controls, are unresponsive to nutritional manipulations that are capable of inducing in normal tissues remarkable modification of specific biochemical activities (1). There are

interesting examples of this defective metabolic regulation in the lipid metabolism of several hepatomas such as the loss of the normal inhibition of cholesterol biosynthesis by dietary cholesterol (2–4) and the lack of adaptation to nutritional manipulations that affect the fatty acid biosynthesis (4–8).

The responsiveness of tumor cells to nutritional manipulations was also assessed by studying the changes in their lipid composition after exposure to essential fatty acid (EFA) deficiency, which induces a typical lipid pattern in normal tissues (9–17) through the induction of adaptive mechanisms (18–22). However studies on the growth of several tumors in EFA-deficient host animals (7, 8, 23–25) have given conflicting results as to the fatty acid composition. This may have been due to differences either in the experimental conditions (time-course of dietetic treatment, etc.) or in the characteristics of the tumor studied (degree of differentiation, solid or ascitic form, etc.).

Therefore it appeared of interest to determine the effect of EFA deficiency on individual lipid classes in a fast-growing, poorly differentiated tumor, the Yoshida ascites hepatoma. Exposure of tumor cells to EFA-deficiency was effected by transplantation of the tumor in adult rats fed an EFA-deficient diet from the time they were weaned. The effects of EFA deficiency on the lipid composition of livers and blood plasma from host rats and normal rats were used as a basis for comparison.

Abbreviations: EFA, essential fatty acids; CE, cholesteryl esters; FC, free cholesterol; TG, triglycerides; FFA, free fatty acids; PLT, total phospholipids; TLC, thin-layer chromatography; PA, phosphatidic acid; DPG, diphosphatidylglycerol (cardiolipin); PE, phosphatidylethanolamine; PI, phosphatidylinositol; PC, phosphatidylcholine; VLDL, very low density lipoprotein.

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MATERIALS AND METHODS

Diets

The EFA-deficient diet used throughout these experiments contained 60% sucrose, 16% fat-free casein, 4% α -cellulose, 1% choline chloride, vitamins, 4% Wesson salt mixture, and 3% hydrogenated beef fat. Cholesterol was not included in this diet. The control diet had the same composition, except that hydrogenated beef fat was replaced by a corresponding amount of 1:1 mixture of corn oil and cod-liver oil. The fatty acid compositions of the two diets are reported in Table 1.

Animals and dietetic treatment

Male Wistar rats at twenty-one days of age were separated into two groups and fed, respectively, the EFA-deficient or the control diet (rich in linoleic acid) for seven weeks. At this point the rats fed the EFA-deficient diet were considered to be deficient in essential fatty acids on the basis of the appearance of the typical scaliness of the skin (9), the disappearance of linoleic acid from the subcutaneous tissue, and the presence of high levels of eicosatrienoic acid in both liver and plasma (9, 10). Contrary to what has been reported by other authors (26), our EFA-deficient rats exhibited a slightly lower body weight than the controls during the whole period of EFA deprivation.

Starting from the end of the seventh week of dietetic treatment, while each group of animals continued to receive its diet, the Yoshida ascites hepatoma (AH 130) was transplanted weekly for five weeks in 3–4 animals of each group. Seven days after the inoculum the rats were fasted overnight and then killed by exsanguination under light diethyl ether anesthesia. Before killing, a blood sample was withdrawn by heart puncture using heparinized syringes, collected in tubes and immediately centrifuged at low speed to separate plasma from red blood cells. At this point the ascitic fluids were collected in refrigerated glassware and immediately centrifuged at 1000 *g* in a Sorvall centrifuge to separate the hepatoma cells from the ascitic plasma. The cells were then washed twice in 0.14 M NaCl and suspended in a known volume of 0.04% CaCl₂. The livers were removed and homogenized in 0.04% CaCl₂. In each experiment, groups of 3–4 rats (fed either the EFA-deficient diet or control diet) that had not been inoculated with Yoshida hepatoma, were processed as the host rats. These animals are referred to as “normal rats” and they received the dietetic treatment for the same length of time as the host rats.

TABLE 1. Fatty acid composition of lipids in EFA-deficient diet and in control diet

Fatty Acid	Control Diet	EFA-Deficient Diet
	<i>% by weight</i>	
Myristic	2.6	2.8
Palmitic	17.2	31.4
Palmitoleic	3.6	
Stearic	4.1	65.8
Oleic	35.1	
Linoleic	32.5	
Linolenic	4.9	

Analysis of lipids

The method of Bligh and Dyer (27) was used to extract the lipids from ascitic plasma and the method of Folch, Lees, and Sloane Stanley (28) was used to extract the lipids from blood plasma, liver homogenates, and cell suspensions.

The extracted lipids were separated into neutral lipids and total phospholipids by silicic acid column chromatography (Unisil 100–200 mesh; Clarkson Chemical Co. Inc., Williamsport, Pa.). Neutral lipids were fractionated into cholesteryl esters (CE), triglycerides (TG), free cholesterol (FC), diglycerides (DG), monoglycerides (MG) and free fatty acids (FFA) by column chromatography on Florisil (100–200 mesh) (Fisher Scientific Co., Fair Lawn, N. J.) (29). Phospholipids of tumor cells and livers were fractionated by silicic acid column chromatography (Unisil 200–325 mesh) into diphosphatidylglycerol (“cardiolipin”, DPG) + phosphatidic acid (PA), phosphatidylethanolamine (PE) + phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylcholine (PC), sphingomyelin (SP) and lysolecithin (LPC) using a stepwise elution system of methanol in chloroform reported in a previous paper (30). PE was then separated from PS on a silicic acid–ammonia silicate column (31).

The identification and purity of the individual lipid classes were ascertained by comparing their chromatographic properties with those of suitable standards on TLC (Silica Gel H, Merck, Darmstadt, West Germany). Hexane–diethyl ether–acetic acid 85:15:1 was used for the TLC of neutral lipids and chloroform–methanol–water 65:25:4 was used for TLC of phospholipids.

Gas–liquid chromatographic analysis

Methyl esters of fatty acids were prepared by refluxing the sample with 5 ml of 5% sulfuric acid in methanol–benzene 25:1. Analysis of methyl esters was performed isothermally at 185°C in an Aerograph model 1520 gas-chromatograph equipped with

double hydrogen flame detector and fitted with 6 ft \times 1/8 in stainless steel columns packed with 15% EGSS-X on 100–200 mesh Gas-Chrom P (Applied Science Laboratories Inc., State College, Pa.). Identities of the major fatty acids were based on pure standards purchased from Applied Science Laboratories and Fluka (Fluka AG, Buchs, Switzerland). Further criteria regarding the degree of unsaturation of the fatty acids were derived from the preliminary fractionation of the fatty acid methyl esters by argentation thin-layer chromatography (32) followed by the gas-liquid chromatographic analysis of the separated subfractions. The unsaturated fatty acids were designated by the trivial name of the more common isomer, although the locations of the double bonds in the hydrocarbon chains were not determined. The eicosatrienoic acid referred to in this paper was the n-9 isomer, which is increased in EFA deficiency. Quantitative responses of the detectors were routinely checked with the NHI-F reference mixture from Applied Science Laboratories.

Analytical methods

The total phospholipids (PLT) and individual phospholipid classes were evaluated from the phosphorus determinations performed by the method of Martin and Doty (33) on the sample digested with H₂SO₄–HClO₄ 3:2. Free cholesterol (FC) and esterified cholesterol (EC) were determined following the method of Cramer and Isaksson (34), glycerol according to the procedure of Carlson (35), and free fatty acids (FFA) by the method of Duncombe (36).

RESULTS

The results reported in the tables are means of values obtained from experiments performed at dif-

ferent times of the dietetic treatments (8th to 12th week). Slight differences in these values did not seem related to the continued exposure to EFA deficiency. Sinclair and Collins (17) noted that after eight weeks there were no further changes in the fatty acid composition in liver from EFA-deficient rats.

Content of water, fat-free dry weight and total lipids in Yoshida hepatoma cells, host liver, and normal liver from rats fed an EFA-deficient diet or a control diet (Table 2)

Yoshida hepatoma cells grown in rats fed the control diet contained more water and a lower concentration of total lipids than livers from normal and host rats fed the same diet. Yoshida hepatoma cells grown in EFA-deficient rats had a lower water content with no appreciable change in the total lipid concentration.

In uninoculated (“normal”) rats, EFA-deficiency resulted in a marked increase of total liver lipids but did not affect the water content. In inoculated (host) EFA-deficient rats there was no increase of liver lipids, but the water content increased slightly compared with the host liver from control rats.

Lipid composition studies

The Yoshida hepatoma cells grown in control rats had a higher level of free cholesterol and lower concentrations of phospholipids, FFA, and TG than the livers of normal rats (Table 3). Similar differences in the content of FFA and free cholesterol were also found when hepatoma cells were compared with the livers from host rats fed the control diet. Host rats fed the control diet had lower levels of liver TG and esterified cholesterol in comparison to the normal rats fed the same diet.

Yoshida hepatoma grown in rats fed the EFA-deficient diet for 8–12 weeks had a decreased con-

TABLE 2. Water, lipid-free dry weight, and total lipids of Yoshida hepatoma cells, host liver and normal liver from rats maintained on a control or EFA-deficient diet

	Yoshida Hepatoma		Host Liver		Normal Liver	
	EFA+ ^a (5) ^b	EFA– (5)	EFA+ (5)	EFA– (5)	EFA+ (4)	EFA– (4)
Water ^c	83.6 \pm 0.27 ^d	78.5 \pm 2.16 ^{*e}	71.5 \pm 0.42	74.5 \pm 0.95 [*]	71.8 \pm 0.73	69.1 \pm 1.55
Lipid-free dry weight ^f	13.9 \pm 0.58	18.7 \pm 2.11	23.8 \pm 0.45	21.3 \pm 1.02	22.3 \pm 1.00	22.2 \pm 1.42
Total lipids ^g	2.3 \pm 0.26	2.8 \pm 0.29	4.6 \pm 0.22	4.0 \pm 0.24	5.7 \pm 0.37	8.0 \pm 0.46 ^{**}

^a EFA+ and EFA– refer, respectively, to the control diet and EFA-deficient diet fed to host rats or normal rats.

^b The numbers in parentheses represent the number of separate experiments performed between the eighth and the twelfth week of the dietetic treatments.

^c The water content was calculated by difference between wet weight and dry constant weight. The wet weight of ascites hepatoma cells was corrected assuming that 17% extracellular water was trapped in the pellet of packed cells.

^d Values are means \pm SEM and are expressed as mg/100 mg wet weight.

^e Level of significant differences between means of EFA+ and EFA– groups: *, **, ***, indicate that means are significantly different, respectively, at $P < 0.05$, < 0.01 and < 0.001 levels.

^f Lipid-free dry weight was calculated by difference between dry weight and total lipids.

^g Total lipids were determined gravimetrically on portions of the lipid extracts evaporated at room temperature under nitrogen.

TABLE 3. Effect of EFA-deficiency on the lipid composition of Yoshida hepatoma cells, and of livers from host and normal rats

	Yoshida Hepatoma		Host Rat Liver		Normal Rat Liver	
	EFA+ ^a (4) ^b	EFA- (4)	EFA+ (5)	EFA- (5)	EFA+ (4)	EFA- (4)
FC (mg) ^c	11.3 ± 0.52	9.9 ± 1.93	6.5 ± 0.51	8.7 ± 0.64 ^{*d}	7.9 ± 0.62	9.7 ± 0.55
EC (mg)	2.7 ± 0.21	1.9 ± 0.38	1.3 ± 0.09	1.2 ± 0.18	2.5 ± 0.44	9.5 ± 1.24 ^{**}
TG (μmoles)	66.1 ± 8.66	93.3 ± 22.58	58.6 ± 3.88	32.7 ± 1.88 ^{***}	122.1 ± 13.72	259.0 ± 22.43 ^{**}
FFA (μmoles)	3.2 ± 0.65	1.3 ± 0.18 [*]	6.4 ± 0.96	6.3 ± 0.90	9.3 ± 0.12	8.9 ± 1.07
PLT (mg) ^e	122.5 ± 12.25	67.5 ± 10.00 [*]	150.0 ± 9.50	152.5 ± 4.75	162.5 ± 4.25	142.5 ± 16.25

^a EFA+ and EFA- refer, respectively, to the control diet and EFA-deficient diet fed to host rats or normal rats.

^b The numbers in parentheses represent the number of separate experiments performed between the eighth and the twelfth week of the dietetic treatments.

^c Values are means ± SEM and refer to the amount of lipid in 1 g of dry lipid-free cells or livers.

^d Level of significant differences between means of EFA+ and EFA- groups: *, **, ***, indicate that means are significantly different respectively at $P < 0.05$, < 0.01 and < 0.001 levels.

^e Total phospholipids = lipid phosphorus × 25.

centration of total phospholipids and FFA; however the concentration of TG in tumor cells was not significantly changed. Livers from uninoculated Wistar rats, fed the EFA-deficient diet for 8–12 weeks showed an increased concentration of TG and esterified cholesterol. The increase of free cholesterol concentration and the reduction of FFA and phospholipid content in the EFA-deficient normal (uninoculated) rats were not significant. Due to EFA deficiency, the liver concentration of diglycerides (not reported in Table 3) was increased in uninoculated rats. The host rats (inoculated) fed the EFA-deficient diet had a lower concentration of TG and a higher level of free cholesterol in the liver than the host rats fed the control diet.

The phospholipids of Yoshida hepatoma cells grown in rats fed the control diet contained 3.8% cardiolipin + phosphatidic acid, 29.1% phosphatidylethanolamine, 4.2% phosphatidylserine, 7.6% phosphatidylinositol, 49.1% phosphatidylcholine, 6.0% sphingomyelin, and 0.6% lysolecithin. A similar com-

position was also found in the livers of host rats and normal rats fed the control diet. There were only minor differences in the phospholipid composition of Yoshida hepatoma cells and livers of host and normal rats in response to feeding an EFA-deficient diet.

The blood plasma of normal rats on the EFA-deficient diet had a lower level of phospholipids than the blood plasma of normal rats fed the control diet. The concentrations of free cholesterol, esterified cholesterol, TG, and FFA of normal rat plasma were not significantly changed by EFA deficiency (Table 4).

Host rats fed a control diet had levels of plasma free cholesterol, esterified cholesterol, and TG higher than normal rats fed the same diet. The levels of plasma diglycerides and monoglycerides (not reported in Table 4) were higher in host rats than in the normal rats fed a control diet. The higher level of plasma FFA in the host rats compared to normal rats fed a control diet was not significant. The host rats fed an EFA-deficient diet showed a noticeable decrease in the concentration of plasma TG and FFA.

TABLE 4. Effect of EFA deficiency on the lipid composition of blood and ascitic plasma from host rats and blood plasma from normal rats

	Host Rat				Normal Rat Blood Plasma	
	Blood Plasma		Ascitic Plasma		EFA+ (4)	EFA- (4)
	EFA+ ^a (5) ^b	EFA- (4)	EFA+ (3)	EFA- (5)		
FC (mg) ^c	31.9 ± 2.58	34.5 ± 1.62	2.5 ± 0.36	6.8 ± 0.97 ^{*d}	15.2 ± 1.55	11.1 ± 0.88
EC (mg)	57.4 ± 4.42	42.4 ± 2.81	19.9 ± 1.03	17.8 ± 2.43	38.0 ± 2.54	35.4 ± 3.17
TG (μmoles)	534.0 ± 54.6	241.2 ± 8.66 ^{**}	8.0 ± 0.57	17.1 ± 5.73	102.4 ± 24.43	105.4 ± 23.08
FFA (μmoles)	114.9 ± 23.28	43.6 ± 6.42 [*]	10.3 ± 1.94	11.9 ± 2.86	81.0 ± 20.02	55.8 ± 11.26
PLT (mg) ^e	137.5 ± 14.00	132.5 ± 10.25	37.0 ± 1.50	24.9 ± 3.59 [*]	130.0 ± 6.50	97.5 ± 3.00 ^{**}

^a EFA+ and EFA- refer, respectively, to the control diet and EFA-deficient diet fed to host rats or normal rats.

^b The numbers in parentheses represent the number of separate experiments performed between the eighth and the twelfth week of the dietetic treatments.

^c Values are means ± SEM and refer to 100 ml blood plasma and ascitic plasma.

^d Level of significant differences between means of EFA+ and EFA- groups: *, **, ***, indicate that means are significantly different, respectively, at $P < 0.05$, < 0.01 and < 0.001 levels.

^e Total phospholipids = lipid phosphorus × 25.

TABLE 5. Fatty acid composition of the main lipid classes of the liver from normal rats fed an EFA-deficient diet (EFA-) or a control diet (EFA+)

Fatty acid ^d	CE		TG		FFA		DPG + PA		PE		PI		PC	
	EFA+ ^a (4) ^b	EFA- (4)	EFA+ (4)	EFA- (4)	EFA+ (4)	EFA- (4)	EFA+ (3)	EFA- (4)	EFA+ (3)	EFA- (3)	EFA+ (3)	EFA- (4)	EFA+ (3)	EFA- (3)
	% (by wt) of total fatty acids ^c													
14:0	0.3	0.3	1.1	1.3	1.4	1.1	1.4	0.6	0.2	0.8	0.4	0.1	0.3	0.3
Ald. 16:0														
16:0	30.9	18.8	28.8	32.7	43.5	40.7	7.8	6.1	21.4	15.4	11.7	9.5	25.8	23.7
16:1	12.0	19.0	10.3	14.2	4.3	8.3	13.0	30.8	1.0	2.4	1.1	1.9	2.1	3.9
Ald. 18:0														
18:0	2.8	3.2	1.9	2.0	12.9	9.8	2.9	1.8	26.1	22.5	43.7	44.8	17.3	17.5
18:1	40.7	56.9	48.1	49.5	21.9	33.9	24.3	41.3	8.7	16.6	6.9	7.9	13.7	23.1
18:2	7.0	0.7	9.1	0.2	8.8	0.6	46.8	13.4	5.1	1.3	3.6		12.5	1.7
20:3	0.3	0.8				4.4	0.1	1.8		18.6		25.1	1.6	22.3
20:4	6.0	0.3	0.7	0.1	7.2	1.3	3.7	2.5	17.7	15.6	19.2	9.3	13.1	5.0
22:2							1.7		2.4	0.8	1.6	0.2	2.3	1.0
22:5									1.0	1.2	0.8		0.5	
22:6									16.4	4.8	10.9	1.2	8.9	1.4
Triene:tetraene ratio ^e	0.05	2.70				3.40	0.03	0.70		1.20		2.70	0.30	4.50

^a EFA+ and EFA- refer, respectively, to the control diet and EFA-deficient diet fed to normal rats.

^b The numbers in parentheses represent the number of separate experiments performed between the eighth and the twelfth week of the dietetic treatments.

^c All the results reported in this table were submitted to the Student's test and only the significant differences are discussed in the text.

^d Fatty acids are designated by chain length:number of double bonds.

^e As determined from the percentage of eicosatrienoic acid and of arachidonic acid reported in the table.

The ascitic plasma from the host rats fed the control diet had low concentrations of lipids; cholesteryl esters and phospholipids were the main constituents. The ascitic plasma from the EFA-deficient host rats had a higher content of free cholesterol and a lower amount of total phospholipids as compared to the ascitic plasma from the control rats. The higher value of the TG concentration found in ascitic plasma from EFA-deficient rats was not significant.

Fatty acid composition of the main lipid classes from the Yoshida hepatoma cells, host liver and normal liver from rats fed either an EFA-deficient diet or a control diet

All the phospholipid classes from livers of EFA-deficient normal rats showed a dramatic reduction of linoleic acid and a concurrent increase of oleic and palmitoleic acids (Table 5). In PE, PI, and PC the eicosatrienoic acid was remarkably increased while arachidonic and docosahexaenoic acids were concomitantly decreased. In PE, as well as in cardiolipin + phosphatidic acid fraction, there was a slightly reduced level of arachidonic acid concomitant with an increased proportion of eicosatrienoic acid. A reduction of linoleic and arachidonic acids and a rise of oleic and palmitoleic acids were also observed in the cholesteryl esters and FFA from livers of EFA-deficient normal rats. Except for a decrease in linoleic acid, the

composition of liver TG from normal rats was barely affected by the EFA-deficient diet.

Tumor-bearing rats (host rats) maintained on an EFA-deficient diet exhibited modifications in the fatty acid composition of the liver phospholipids and neutral lipids similar to those observed in normal rats fed the same diet, with the exception that in the latter animals there was more C_{20:3} in phosphatidylcholine and less in phosphatidylethanolamine (Table 6).

The fatty acid composition of the individual lipid classes from Yoshida hepatoma cells grown in rats fed a control diet showed a rather typical pattern, different from that found in the corresponding classes of livers from both host rats and normal rats fed the same diet (Table 7). In fact, PE, PI, and PC of Yoshida hepatoma cells had higher levels of palmitoleic and oleic acids and lower proportions of stearic, arachidonic and docosahexaenoic acids than the same phospholipid classes of the liver of normal and host rats. In addition, PE of tumor cells showed a lower proportion of palmitic acid, an increased level of linoleic acid, and appreciable quantities of C₁₆ and C₁₈ aldehydes in comparison to host and normal liver. Cardiolipin + phosphatidic acid of hepatoma cells contained a higher proportion of oleic acid and a lower proportion of linoleic acid than the host and normal rat livers. In comparison to the normal and host rat livers, tumor cells also showed a lower level of palmitic acid and a higher proportion of oleic acid

TABLE 6. Fatty acid composition of the main lipid classes of the liver from host rats fed an EFA-deficient diet (EFA-) or a control diet (EFA+)

Fatty acid ^d	CE		TG		FFA		DPG + PA		PE		PI		PC	
	EFA+ ^a (5) ^b	EFA- (5)	EFA+ (5)	EFA- (5)	EFA+ (5)	EFA- (5)	EFA+ (3)	EFA- (4)	EFA+ (3)	EFA- (3)	EFA+ (3)	EFA- (4)	EFA+ (3)	EFA- (3)
	%(by wt) of total fatty acids ^c													
14:0	0.6	0.2	0.7	0.9	1.6	1.2	0.3	1.2	0.2	0.5	0.9	0.9	0.5	0.9
Ald. 16:0														
16:0	39.9	42.0	32.2	32.8	52.3	45.9	7.4	9.1	20.8	18.5	13.4	13.6	29.8	34.2
16:1	5.2	5.8	4.7	4.5	2.4	3.0	6.9	21.5	0.4	2.2	1.1	1.2	1.2	3.5
Ald. 18:0														
18:0	4.1	4.9	2.6	2.8	11.2	12.6	4.2	5.0	26.4	23.9	38.9	38.4	19.6	16.1
18:1	28.0	38.8	43.3	56.5	15.4	30.1	22.1	46.0	3.9	14.9	5.1	7.9	11.0	26.8
18:2	8.2	1.2	10.4	0.4	6.6	1.5	52.2	14.3	3.8	1.7	3.0	0.5	9.8	1.7
20:3	0.4	6.6	0.1	2.1	0.1	4.9	4.5	1.5	1.0	24.5	0.8	26.4	0.9	13.9
20:4	12.9	0.5	3.7		10.4	0.8	2.4	1.4	18.5	10.4	22.4	10.2	14.8	2.3
22:2	0.7		0.4						1.9	0.2	1.2		0.8	0.6
22:5			0.5						2.2		2.7		0.5	
22:6			1.4						20.9	3.2	10.6		11.1	
Triene:tetraene ratio ^e	0.03	13.20	0.03		0.01	6.10	1.80	1.10	0.05	2.40	0.04	2.60	0.06	6.00

^a EFA+ and EFA- refer, respectively, to the control diet and EFA-deficient diet fed to host rats.

^b The numbers in parentheses represent the number of separate experiments performed between the eighth and the twelfth week of the dietetic treatments.

^c All the results reported in this table were submitted to the Student's test and only the significant differences are discussed in the text.

^d Fatty acids are designated by chain length:number of double bonds.

^e As determined from the percentage of eicosatrienoic acid and of arachidonic acid reported in the table.

in cholesteryl esters, and a lower proportion of palmitic acid and a higher level of stearic acid in TG and FFA.

Growth of tumor cells in EFA-deficient host rats resulted in an extreme reduction of linoleic acid in

both neutral lipids and phospholipids and a remarkable increase of oleic and palmitoleic acids mainly in cardiolipin + phosphatidic acid, PE, PI, PC, and FFA. Oleic acid was slightly but significantly increased in cholesteryl esters and TG of Yoshida hepatoma cells

TABLE 7. Fatty acid composition of the main lipid classes of the Yoshida hepatoma cells grown in EFA-deficient rats (EFA-) or in control rats (EFA+)

Fatty acid ^d	CE		TG		FFA		DPG + PA		PE		PI		PC	
	EFA+ ^a (5) ^b	EFA- (4)	EFA+ (5)	EFA- (3)	EFA+ (5)	EFA- (4)	EFA+ (3)	EFA- (3)	EFA+ (3)	EFA- (4)	EFA+ (3)	EFA- (4)	EFA+ (3)	EFA- (4)
	%(by wt) of total fatty acids ^c													
14:0	1.4	3.4	3.4	1.0	4.0	3.0	1.7	0.8	0.4	0.3	0.8	1.0	1.4	1.0
Ald 16:0														
16:0	17.0	15.2	22.5	21.7	33.5	31.1	10.2	10.9	10.2	9.7	10.0	10.5	27.8	23.4
16:1	6.6	8.2	5.3	9.9	4.1	7.2	10.8	22.0	2.6	5.4	2.8	3.9	5.4	7.3
Ald. 18:0														
18:0	1.0								1.9	2.3				0.6
18:1	6.8	5.0	8.6	6.0	20.9	15.8	7.0	6.1	15.8	11.8	33.6	28.2	9.5	8.4
18:2	50.0	56.3	46.2	57.9	26.4	40.6	32.6	44.9	27.6	46.7	25.6	35.7	36.0	51.6
18:3	5.8	1.6	11.4	1.9	5.3	1.8	33.2	6.5	12.7	2.5	7.5	0.8	10.6	1.0
20:3	1.6	6.7	0.9	1.2		0.5		5.3	0.6	11.5	1.1	15.1	0.7	3.0
20:4	8.6	3.0	3.7	0.4	5.8				2.3	3.6	13.8	3.7	14.6	2.8
22:2									1.4		3.7	1.1	1.7	0.7
22:5											1.6	0.3	1.5	0.6
22:6									0.8		6.7	1.1	2.5	0.3
Triene:tetraene ratio ^e	0.20	2.20	0.20	3.00					1.50	0.04	3.10	0.08	5.40	0.10

^a EFA+ and EFA- refer, respectively, to the control diet and EFA-deficient diet fed to host rats.

^b The numbers in parentheses represent the number of separate experiments performed between the eighth and the twelfth week of the dietetic treatments.

^c All the results reported in this table were submitted to the Student's test and only the significant differences are discussed in the text.

^d Fatty acids are designated by chain length:number of double bonds.

^e As determined from the percentage of eicosatrienoic acid and of arachidonic acid reported in the table.

TABLE 8. Fatty acid composition of the major lipid classes of blood plasma from normal rats fed an EFA-deficient diet (EFA-) or a control diet (EFA+)

Fatty acid ^d	CE		TG		FFA		PLT	
	EFA+ ^a	EFA-	EFA+	EFA-	EFA+	EFA-	EFA+	EFA-
	(4) ^b	(4)	(3)	(4)	(4)	(4)	(4)	(3)
	<i>%(by wt) of total fatty acids^c</i>							
14:0	0.6		1.3	0.2	2.2	1.7	0.2	
16:0	17.6	6.9	29.2	26.4	30.2	35.7	25.2	19.5
16:1	9.8	14.2	7.2	10.5	12.0	13.5	1.0	1.9
18:0	1.4	0.8	2.8	3.2	4.9	6.8	20.3	23.9
18:1	30.5	30.5	45.3	57.8	32.8	39.9	12.7	20.1
18:2	15.3	3.4	10.6	0.6	7.4	0.9	14.4	1.9
20:3		35.3		1.0		1.5	1.9	25.9
20:4	22.1	8.9	0.4	0.3	10.5		12.8	6.8
22:2	2.7		0.9				1.2	
22:5			0.4				0.8	
22:6			1.9				7.6	
Others							1.9	
Triene:tetraene ratio ^e		4.00		3.30			0.14	3.80

^a EFA+ and EFA- refer, respectively, to the control diet and EFA-deficient diet fed to normal rats.

^b The numbers in parentheses represent the number of separate experiments performed between the eighth and the twelfth week of the dietetic treatments.

^c All the results reported in this table were submitted to the Student's test and only the significant differences are discussed in the text.

^d Fatty acids are designated by chain length:number of double bonds.

^e As determined from the percentage of eicosatrienoic acid and of arachidonic acid reported in the table.

grown in EFA-deficient rats. In addition, the level of eicosatrienoic acid was increased in the cholesteryl esters, and in the PE, PC, and PI from tumor cells grown in EFA-deficient rats. In cardiolipin + phosphatidic acid an increased level of eicosatrienoic acid was concomitant with an unchanged proportion of arachidonic acid. The low level of C₂₂ polyunsaturated fatty acids in the phospholipids of tumor cells grown in control rats was even lower when the tumor cells were grown in EFA-deficient animals.

In Yoshida hepatoma cells, as well as in livers of host and normal rats, there were also changes in the fatty acid composition of phosphatidylserine and lysolecithin similar to those reported for the other phospholipids, while sphingomyelin was scarcely affected by EFA deficiency (results not reported in the tables).

The triene:tetraene ratio in the various lipid classes, taken as a biochemical index of EFA deficiency (37), was comparable in Yoshida hepatoma cells, in host liver, and in normal liver from EFA-deficient rats (Tables 5-7).

Fatty acid composition of the major lipid classes of blood plasma from normal rats and of blood and ascitic plasmas from host rats fed an EFA-deficient diet or a control diet (Tables 8, 9)

In the blood plasma phospholipids of EFA-deficient normal rats there were reductions of linoleic, arach-

idonic, and C₂₂ polyunsaturated fatty acids associated with an increase of oleic, palmitoleic, and eicosatrienoic acids (Table 8). In the blood plasma cholesteryl esters of normal rats, EFA deficiency resulted in a reduced content of palmitic, linoleic, and arachidonic acids, and an increased proportion of palmitoleic and eicosatrienoic acids; however, the level of oleic acid was unaffected. The blood plasma FFA and triglycerides of EFA-deficient normal rats showed a decrease of linoleic acid, a disappearance of arachidonic acid, and an increase of oleic acid.

The blood plasma of host rats fed a control diet, in comparison to the blood plasma of the normal rats fed the same diet, had a lower proportion of oleic acid and a higher proportion of arachidonic acid in cholesteryl esters and a considerably lower level of arachidonic acid in FFA (Table 9). The fatty acid composition of the lipids of the ascitic plasma from host rats fed a control diet reflected the composition of blood plasma with the exception of a lower proportion of oleic acid and a higher level of arachidonic acid in cholesteryl esters.

Blood plasma and ascitic plasma from host rats fed an EFA-deficient diet showed changes in the fatty acid composition similar to those observed in the blood plasma from EFA-deficient normal rats except for an increased level of oleic acid in the cholesteryl esters.

The triene:tetraene ratio was similarly increased in

TABLE 9. Fatty acid composition of the major lipid classes of blood plasma and ascitic plasma from host rats fed an EFA-deficient diet (EFA-) or a control diet (EFA+)

Fatty acid ^d	Blood Plasma						Ascitic Plasma									
	CE		TG		FFA		PLT		CE		TG		FFA		PLT	
	EFA+ ^a (5) ^b	EFA- (5)	EFA+ (5)	EFA- (4)	EFA+ (5)	EFA- (5)	EFA+ (5)	EFA- (4)	EFA+ (3)	EFA- (3)	EFA+ (3)	EFA- (5)	EFA+ (3)	EFA- (5)	EFA+ (3)	EFA- (5)
	% (by wt) of total fatty acids ^c															
14:0	0.1	0.3	0.8	0.1	2.1	0.7	0.7	0.2	0.5	0.6	0.2	0.5	0.6	2.4	2.5	0.1
16:0	17.3	11.3	28.7	32.7	38.0	29.9	20.0	24.6	10.5	8.9	28.4	26.9	30.6	31.4	32.7	26.7
16:1	4.8	9.0	4.7	4.7	9.4	9.4	0.2	1.5	5.6	14.0	7.4	6.5	9.0	12.9	1.0	2.2
18:0	2.9	1.2	3.3	5.0	3.2	6.7	17.9	22.8	0.4	0.6	3.7	3.4	11.3	6.4	17.8	22.6
18:1	22.5	42.8	40.3	53.2	38.5	51.6	9.8	24.0	13.9	42.1	45.2	59.7	38.1	45.5	12.8	27.8
18:2	19.6	4.6	13.3	0.7	8.5	0.2	13.7	3.6	18.5	4.9	11.8	0.3	7.3	0.8	10.5	2.2
20:3	0.7	23.1	0.2	2.8	1.1	1.1	2.1	19.4	0.9	23.0	0.4	2.1	0.3	0.3	2.1	14.4
20:4	32.1	7.7	5.9	0.8	0.3	0.4	16.4	4.1	46.1	6.3	2.6	0.5	1.3	0.2	10.8	3.1
22:2			0.2		0.6		0.6		3.4						0.3	
22:5			0.5		1.6		1.6								0.8	
22:6			2.1		16.3		16.3								7.9	
Others					1.4		1.4								2.9	
Triene:tetraene ratio ^e	0.02	3.00	0.03	3.50	2.80	2.80	0.10	4.70	0.02	3.70	0.20	4.20	1.50	1.50	0.20	4.60

^a EFA+ and EFA- refer, respectively, to the control diet and EFA-deficient diet fed to host rats.
^b The numbers in parentheses represent the number of separate experiments performed between the eighth and the twelfth week of the dietetic treatments.

^c All the results reported in this table were submitted to the Student's test and only the significant differences are discussed in the text.
^d Fatty acids are designated by chain length: number of double bonds.

^e As determined from the percentage of eicosatrienoic acid and of arachidonic acid reported in the table.

the lipids of blood plasma from normal rats and in the lipids of blood and ascitic plasmas from host rats after they had been maintained on an EFA-deficient diet.

DISCUSSION

In the present investigation, growth of the Yoshida hepatoma in EFA-deficient animals was used as a means to assess whether the adaptive response to nutritional manipulation is maintained in a poorly differentiated tumor.

The animals used for tumor transplantation exhibited characteristic features of EFA deficiency as shown by the lipid analyses carried out weekly on uninoculated rats from the 8th through the 12th week of EFA-deficient dietary regime. These features consisted of an increase of liver cholesteryl esters and TG, a reduction of plasma phospholipids, and the occurrence in both liver and plasma of a fatty acid pattern that is considered typical of EFA deficiency (9–17). In the plasma of these rats we did not find the increase of FFA (16–17) and the decrease of TG (15, 17, 38) observed by other investigators during EFA deficiency. These discrepancies may be due to many factors, e.g., the strain of rats, the procedure used to induce EFA deficiency (fat-free diet or diet containing hydrogenated fat), the duration of the dietetic treatment, and the nutritional state of the animals before death.

Regardless of diet, host rats exhibited differences in the liver and plasma lipid composition when compared to the normal rats. In the host rats fed a control diet there was a lower content of TG and esterified cholesterol in the liver and higher levels of TG, free cholesterol, and esterified cholesterol in the plasma than in normal rats fed the same diet. Host rats fed an EFA-deficient diet, unlike normal EFA-deficient rats, had a lower content of liver TG, probably due to the interference of the tumor on the multifactorial mechanism that underlies the fat accumulation in the liver during EFA deficiency (16, 17, 20, 21, 39, 40).

The plasma TG level of host rats, markedly high under normal dietetic conditions, was decreased when host rats were fed an EFA-deficient diet, although it was still higher than that found in EFA-deficient normal rats. This decrease may be related to the reduced level of plasma FFA observed in the EFA-deficient host rats or, alternatively, to an increased lipoprotein lipase activity. With regard to this latter possibility, De Pury and Collins (41) have noticed a relationship between the lipoprotein lipase activity and the VLDL level in the plasma of EFA-deficient rats. However, under our conditions, normal rats kept

on an EFA-deficient diet did not exhibit a decreased level of plasma TG.

Comparison between host rats and normal rats also showed that the presence of hepatoma in animals fed a control diet has an influence on the fatty acid composition of plasma lipids, but does not influence the typical fatty acid pattern in the liver and plasma lipids of the animals fed an EFA-deficient diet.

Yoshida hepatoma cells grown in rats fed an EFA-supplemented diet contained a lower amount of FFA and total phospholipids and a higher level of free cholesterol in comparison to the liver of normal and host rats. In addition, the main phospholipid classes in the hepatoma cells showed a higher proportion of oleic and palmitoleic acids, a lower level of palmitic, stearic, arachidonic and C₂₂ polyunsaturated fatty acids and rather significant proportions of plasmalogenic chains. This fatty acid pattern has previously been found in the same hepatoma (30, 42) and in several other neoplasms maintained in animals fed a stock diet (43–45), and in a hepatoma grown in tissue culture (46).

Growth of Yoshida hepatoma in EFA-deficient rats resulted in a decrease of FFA and total phospholipids and in changes of the fatty acid composition typical of EFA deficiency. In fact, in the ethanolamine, inositol and choline glycerophospholipids of Yoshida hepatoma cells grown in the EFA-deficient rats, there was a decrease of linoleic, arachidonic and docosahexaenoic acids and an increase of oleic and palmitoleic acids. These latter reached a level much higher than that observed in the hepatoma cells grown in animals fed a control diet. In addition the proportion of eicosatrienoic acid was increased, although this response was less evident than in the liver of normal and host rats. These modifications in the fatty acid pattern suggest that the poorly differentiated, fast-growing Yoshida hepatoma is capable of an adaptive response similar to that exhibited by normal tissues during EFA deficiency. In particular the occurrence of eicosatrienoic acid suggests a shift of the polyunsaturated fatty acid synthesis from the n-6 to the n-9 series (10, 18), although it cannot be ruled out that the host may have been the source of the eicosatrienoic acid. In this regard, it should be mentioned that Dunbar and Bailey reported that Ehrlich carcinoma and sarcoma 180 failed to show the occurrence of eicosatrienoic acid when cultured in an EFA-deficient environment (47). This was taken as evidence that tumor cells lack the capacity of synthesizing polyunsaturated fatty acids and that the different series of these fatty acids found in tumors are derived from the host. On the other hand, the results of investigations recently carried out in our laboratory show that SV40-transformed

3T3 cells grown in media containing delipidized fetal calf serum do contain eicosatrienoic acid in their major glycerophospholipids, although at a lower level than normal 3T3 cells grown in the same media. The reduced increase of eicosatrienoic acid observed in tumor cells in vitro in response to EFA deficiency is consistent with the low level of this fatty acid found in Yoshida hepatoma cells grown in EFA-deficient animals, and may be tentatively related to the low activity found in several neoplasms (48, 49) of Δ -6 desaturase, a key enzyme in the biosynthesis of the different series of polyunsaturated fatty acids (50).

After the present paper was submitted for publication, there were reports by R. Wood (51) and by G. A. Rao and S. Abraham (52) showing alterations in the fatty acid composition of individual phospholipid classes in "minimal deviation" 7288c hepatoma (51) and mouse mammary carcinoma (52) grown in EFA-deficient animals. The response of these tumors to EFA deficiency appears to be less pronounced than that of Yoshida hepatoma as shown in this paper. A possible explanation of these differences is that the ascitic condition and the rapid growth rate of the Yoshida hepatoma used in this investigation make this tumor more susceptible to EFA deficiency. ■

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