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Modulation of CD4 expression on lymphoma cells transplanted to mice fed ($n-3$) polyunsaturated fatty acids

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Groups of adult AKR mice were fed well defined fats controlled diet regimens. These consisted of either saturated (beef tallow: 'BT') or ($n-3$) polyunsaturated (fish oil: 'FO') fatty acids supplementation to basal mix mouse food. In other groups, the basal mix was given without any fat supplement ('NF'). Six weeks or more after the initiation of these diet regimens, mice received intraperitoneal injection of histocompatible RDM-4 lymphoma cells. Ascites RDM-4 tumors were harvested approximately two weeks later, and some of their physicochemical properties were studied. It was repeatedly found that: (1) the tumor grew considerably faster in the FO-fed donor than in the BT- or NF-fed donors; (2) cell membrane fluidity, content of $C_{20}(n-3)$ and of $C_{22}(n-3)$ fatty acids were significantly higher in the FO groups than in both BT and NF groups, while the content of $C_{20}(n-6)$ and $22:4(n-6)$ fatty acids was concomitantly decreased; (3) expression of the CD4 cell surface marker was always significantly diminished in the FO groups, whereas other markers such as CD8, H2K, Thy-1 and LFA-1 were not affected. Similar results were obtained, whether fats constituted from 1% to 16% by weight of the food intake. Use of a recently selected line of the RDM-4 lymphoma, exhibiting higher CD4 marker expression, resulted in similar observations. On the other hand, CD4 expression on cells from lymphoid organs of healthy adult AKR mice was not detectably modulated by the dietary fats.

Introduction

Physicochemical properties of eukaryote cell membranes play important roles in cell physiologic functions [1]. They can be significantly modulated by the composition of dietary fats [2] especially in the case of lymphoid tumors growing in the peritoneal cavity [3]. Thus, the observations that dietary fat composition may alter host-tumor relationships were not unexpected [4]. We recently observed that RDM-4, an AKR mouse strain lymphoma, after being transplanted intraperitoneally into syngeneic recipients, grew consistently faster in mice fed fish oil (FO) than in mice fed beef tallow (BT) [5]. These changes correlated with significant differences in saturation levels of the fatty acids.

In addition, RDM-4 cells harvested from donors fed FO, whose membrane phospholipids were richer in ($n-3$) unsaturated fatty acids than those of lymphoma cells harvested from donors fed BT, exhibited a significantly higher resistance to lysis by non specific antitumor effectors Lymphokine Activated Killer (LAK) cells [5]. Target cell membrane structures that are recognized by the lytic LAK cells have not yet been formally identified. 'Cold target inhibition' experiments suggested that the expression of these membrane structures on RDM-4 lymphoma cells can be modulated by certain antibiotics treatments [6], under conditions whereby lipid composition, as well as cell membrane fluidity and permeability, were profoundly affected [7]. On the other hand, it has been shown that changes in the membrane fatty acid saturation may alter considerably, but in an apparently highly selective fashion, the expression of some membrane markers, as recently revealed by flow cytofluorometric studies [8].

In the present article we show that the nature of the dietary fatty acids (saturation and ($n-3$) unsaturation)

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given to mice bearing ascitic RDM-4 lymphoma, influences some physicochemical properties of the lymphoma cell membranes, as well as the quantitative expression of the CD4 marker.

Materials and Methods

Tumor system

The RDM-4 lymphoma cells were passaged either by intraperitoneal transplantations in syngeneic AKR mice, or cultured in RPMI 1640 medium supplemented with fetal calf serum (FCS) and antibiotics as described elsewhere [6]. Cultured RDM4 cells are CD4 negative [9] and acquire the CD4 marker as they expand in vivo. This acquisition process is rather slow, since the percentage of CD4 positive cells is $\cong 10\%$ after one week of intraperitoneal growth, and $\cong 30\%$ after two weeks (unpublished). In addition, it was cloned in vitro by seeding in 96 wells culture trays (0.1 ml per well) at the concentration of 10–20 cells/ml with irradiated spleen feeder cells. Several clones were expanded in vitro and transplanted intraperitoneally into AKR mice, harvested after a 2 week in vivo expansion and tested for CD4 by cytofluorometry. One of these, thereafter called RDM4-L1, was selected on the basis of its highest frequency of CD4 positive cells at the time of harvesting. For experimental use of RDM-4-L1, an aliquot of $2 \cdot 10^6$ cells was thawed and expanded in culture for 14 days before being injected intraperitoneally to AKR mice of the desired groups. For each experimental group, RDM-4 cells were injected 10^5 cells per mouse, and harvested by peritoneal lavage after 12–15 days. Each experimental group consisted of 2–4 tumor donor mice.

Diet regimens

AKR mice received special diets 6 weeks or more before receiving the tumor graft. The diets consisted of 'basal mix for adjusted fat' (No. TD86145 Teklad®, Madison, WI) to which proportions of either 16%, 8%, 6%, 4% and 1% (by weight) fats were added. Fats consisted of either fish oil (FO) from Efamol Institute, Kentville, N.S., Canada, or beef-tallow (BT) (from ICN, Montreal, Canada). FO consists mainly of 23.7% saturated fatty acids and of PUFA of the $(n-3)$ series (20 : 5, 16.8%; 22 : 6, 11.1%; 22 : 5, 2.4%) linoleic acid (1.3%) and other monounsaturated fatty acids of the $(n-7)$ and $(n-9)$ series [5]. BT consists of mainly 47.8% saturated fatty acids (14 : 0, 6.3%, 16 : 0, 27.4%, 18 : 0, 14.1%), 49.6% monounsaturated oleic acid (18 : 1$(n-9)$) and 2.5% linoleic acid (18 : 2$(n-6)$). Proportion of 8%, 10%, 12% and 15% (by weight) sucrose were added to the preparations containing 8%, 6%, 4% and 1% fats, respectively, to keep constant the mass intake in all the groups. In the same way, a 'no fat' (NF) group received 16% sucrose supplementation to 'basal mix'. FO was stored refrigerated under nitrogen atmosphere. New batches of food were prepared on a weekly basis. Cholesterol contents of the different preparations were determined and, when necessary, readjustment of the cholesterol was made to ensure uniformity of this parameter in all the experimental groups. For comparison, some other donors were fed regular laboratory mouse chow (Prolab, Agway, Syracuse, NY).

Cell preparations

Peritoneal ascites cells, obtained by lavages, were washed twice in Earle's basal saline solution (EBSS). They were incubated 1 h in RPMI 1640 medium, supplemented as described [6], at 37 °C in plastic dishes, in order to eliminate adherent macrophages. They were then counted in a hemocytometer, after trypan blue staining, and the number of viable RDM-4 cells per peritoneum was calculated. Harvested RDM-4 cells from different mice of the same diet groups were either pooled or used separately. They were used for cytofluorometric evaluation of cell surface markers, for membrane fluidity determination (fluorescence polarization of TMA-DPH probe) and for chemical analyses of fatty acids composition. Spleens, lymph nodes and thymuses from tumor-free adult AKR mice were removed from freshly killed donors and the lymphoid cells suspensions were prepared as usual [6] before cytofluorometer determinations of the CD4 marker on their surface. Cell viability always exceeded 95%, as judged by trypan blue exclusion test.

Quantitative surface markers determination

(i) Staining of surface antigens. RDM-4 cells were directly labelled for immunofluorescence. Cells ($3 \cdot 10^6$) were centrifuged and pellets were resuspended in 30 μl of the following antibodies: phycoerythin (PE) conjugated anti-L3T4 (CD4) (Beckton-Dickinson, Mountain View, CA, U.S.A.) and fluorescein isothiocyanate (FITC) conjugated anti-Lyt-2 (CD8) (hybridoma 53-6-72, ATCC), anti-Thy1.1 (T11D7e2, ATCC), anti-H2K^k (Becton-Dickinson) and anti-LFA-1 (clone I21.7.7, courtesy of Dr. Trevor Owens, Mc Gill University, Montreal). The suspensions were incubated for 45 min at 4 °C. Cells were then washed through a layer of fetal calf serum (Flow) in order to remove free antibody. Cells were washed three times in PBS containing 0.1% NaN_3 and 1% FCS, centrifuged and their concentration adjusted to 10^6/ml.

(ii) Flow cytometry. Cells were analysed for distributions and for either one or two colors fluorescence, using an EPICS C cytofluorometer (Coulter Electronics, Hialeah, FL, U.S.A.) equipped with a single Argon laser emitting at 488 nm and with two logarithmic (three decades) intensity scales. Dead cells (less than 5%) were routinely excluded from the analyses on the basis of forward light scatter. Detection of FITC and PE was done with photomultipliers of 520 and 560 nm, respec-

tively. In each case, profiles obtained with the appropriate controls were deducted from profiles of stained cells.

Membrane fluidity determination

1-(4-Trimethylammoniumphenyl)-6-phenyl-1,3,5-hexatriene, *p*-toluenesulfonate (TMA-DPH), purchased from Molecular Probes (Eugene, OR), was chosen as the fluorescence probe due to its specific localization to the plasma membrane [10]. TMA-DPH was prepared as a stock solution of $2 \cdot 10^{-3}$ M in dimethyl formamide and diluted in phosphate-buffered saline (PBS) under vigorous stirring to $1 \cdot 10^{-5}$ M just prior to use. RDM-4 cells were washed twice with PBS and finally suspended at a concentration of $1 \cdot 10^{6}$ cells/ml in PBS containing $1 \cdot 10^{-6}$ M TMA-DPH. The cells were incubated for 45 min at 37° C, centrifuged for 10 min to eliminate excess probe and resuspended in PBS. Fluorescence intensities were measured on a Shimazu RF-540 spectrofluorometer, using excitation and emission wavelengths of 360 nm and 425 nm, respectively. The fluorescence polarization ratio was calculated according to the following equation:

$$P = \frac{I_{ee} - I_{eb}(I_{be}/I_{bb})}{I_{ee} + I_{eb}(I_{be}/I_{bb})}$$

where e and b represent the position of the polarizers at which only the parallell or the vertical components of the light beam are transmitted.

Chemical analysis of fatty acids compositions

Pellets of $50 \cdot 10^{6}$ cells were resuspended in 1 ml PBS. The lipids were extracted by adding 4 ml chloroform and 2 ml methanol to the suspension. The mixture was sonicated and incubated overnight at 4° C. The bottom layer was removed and evaporated. The lipid extract was dissolved in 1 ml chloroform. 0.1 ml was used for total lipid analysis and the remaining 0.9 ml used for lipid fractionation on thin-layer chromatography. The fatty acids of the total lipid or of the phospholipid fractions were transesterified using boron trifluoride in methanol (14% w/v) as described by Manku et al. [11] and the fatty acid methyl esters analyzed by gas chromatography as previously described [7].

Results

Tumor growth in vivo

We repeatedly confirmed the finding that tumor donors fed FO yielded more lymphoma ascites cells, upon peritoneal lavages performed about two weeks post grafting. It is striking that this effect was still marked in the groups in which the proportion of dietary fat consisted of only 1%. Thus, in a typical experiment, peritoneal lavages of RDM-4-L1 yielded $50 \cdot 10^{7}$ cells when harvested from donors fed 1% FO, versus $17 \cdot 10^{7}$ cells from their experimental counterparts fed 1% BT, and $14 \cdot 10^{7}$ cells from donors fed 'no fat' regimen (means of two mice per group).

Cell markers expression and modulation

In two experiments using unselected RDM-4 cells, we observed that the CD8, $H2K^{k}$, Thy.1 and LFA-1 markers remained at the same levels (1–2%, 91–98%, 90–97% and 91–97%, respectively) whatever the diet given to the tumor bearers. In contrast, the CD4 positive population was significantly decreased (approximately from 30% to 15%) when RDM-4 cells were harvested from donors fed FO, compared to donors fed either BT or regular chow of commercial origin. This observation was made whatever the proportion of fat (16%, 8%, 4%) in the diet (results not shown).

In another experiment, RDM-4-L1 was used and it was confirmed that the expression of CD4, but not that of CD8, was constantly lower (from ≅ 65% to ≅ 50%) when the tumor was harvested from donors fed 16%, 8% and 4% FO, compared with donor fed regular chow (not shown).

In two subsequent experiments, RDM-4-L1 cells were used and the donor mice were fed diets of normal and

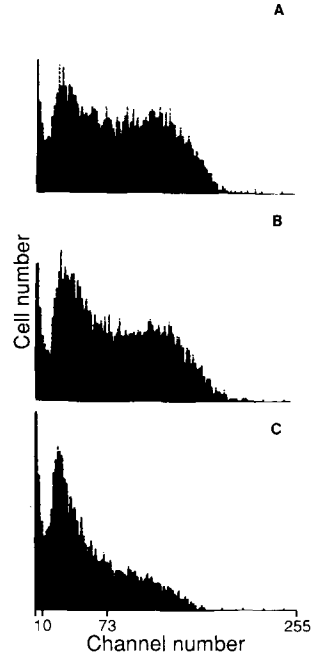

Fig. 1. Cytofluorographs of PE-conjugated anti-L3T4 (CD4) monoclonal antibody on RDM-4-L1 lymphoma cells harvested from syngeneic AKR donors fed: (A) no fat; (B) beef tallow (6% by weight of food intake); (C) fish oil (6% by weight of food intake). Ordinate: cell number per channel; abcissa: channel number. 'High' fluorescence refers to fluorescence recorded in channel numbers 73 and beyond, whereas 'low' fluorescence refers to channel numbers between 10 and 73. Fluorescence recorded in channels 1–9 was considered negative. For each group, lymphoma cells from two donor mice were pooled. Similar results were obtained with the 1% fat groups.

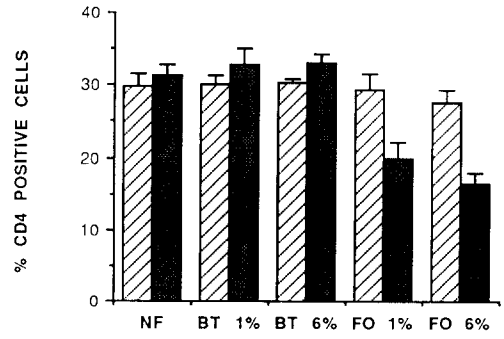

Fig. 2. Percentage of L3T4(CD4)-positive RDM-4-L1 lymphoma cells harvested from donors fed: NF, no fat; BT 1%, beef tallow (1% by weight of food intake); BT 6%, beef tallow (6% by weight of food intake); FO 1%, fish oil (1% by weight of food intake); FO 6%, fish oil (6% by weight of food intake). Hatched bars: percentage of cells exhibiting 'low' fluorescence intensity. Solid bars: percentage of cells exhibiting 'high' fluorescence intensity. Each value represents the means (±standard deviation) obtained from four lymphoma cells donors. Student's *t*-test analysis revealed a significant ($P < 0.05$) difference between the FO groups and the BT or NF groups, in the case of the 'high' fluorescence intensity.

low fat compositions, namely 6% and 1%. Controls consisted of groups fed 'no fat'.

In all experimental groups, it was confirmed that the proportion of CD4 positive RDM-4-L1 cells was always reduced by approx. 15–20% when harvested from donors fed FO (either 1% or 6%). The printout charts of the cytofluometer depict the existence of different levels in the red fluorescence intensity of the PE-conjugated anti-CD4 monoclonal antibody, and we distinguished the 'high' from the 'low' fluorescing cells, using channel number 73 as cutoff point. It can be seen that the 'high' fluorescence intensity is the only one which was modulated, in the RDM-4-L1 cells harvested from FO fed donors (Fig. 1). The same result has been found in another experiment involving four mice per group, individually tested (Fig. 2). In no instance the RDM-4-L1 cell size distributions were affected by the diet of the tumor donors (result not shown).

The thymuses, lymph nodes and spleens of AKR mice fed BT (1% and 6%), FO (1% and 6%), NF or chow for 6 weeks were harvested. Lymphocyte suspensions were prepared and stained with PE anti-L3T4 (CD4) antibodies, as described in Material and Methods. For each organ, regardless of the diet, cytofluorographs of such preparations were identical in the proportion of stained cells and in the intensity of staining with the antibodies. In short, no deviation from the classical pattern was found in any group (Table I).

Membrane fluidity

The polarization data (Fig. 3) show that TMA-DPH incorporated in RDM4-L1 cells harvested from mice fed FO exhibited significantly lower polarization values, which corresponds to higher membrane fluidity. On the other hand, no changes could be observed in cells harvested from mice fed BT, as compared to the 'no fat' controls. Similar results were obtained for mice fed a diet containing either 1% or 6% of the same type of fats.

TABLE I

Percentage of CD4 positive cells in lymphoid organs of healthy adult AKR mice

Figures are presented as means ± S.D. of three donor mice.

Diet	Spleen	Lymph node	Thymus
FO 1%	22.3 ± 4.0	53.6 ± 1.5	93.3 ± 0.6
FO 6%	23.3 ± 4.0	56.6 ± 0.6	96.3 ± 0.6
BT 1%	29.0 ± 4.6	61.6 ± 1.5	93.7 ± 0.6
BT 6%	26.7 ± 2.5	58.0 ± 2.8	95.0 ± 0.6
NF	25.3 ± 0.6	56.3 ± 0.6	95.7 ± 0.6
Chow	25.0 ± 2.6	59.3 ± 2.9	95.7 ± 0.6

Membrane fatty acid composition

The fatty acid composition of RDM-4-L1 cells grown in mice fed on 1% FO diet differed from those fed BT 1% diet (Table II). The levels of C_{20} and C_{22} ($n-3$) fatty acids in both total lipid and phospholipid fractions increased dramatically in RDM-4-L1 cells grown in FO-fed mice, compared to those grown in BT-fed mice. The increase in C_{20} and C_{22} ($n-3$) fatty acids was concomitant with a sharp decrease in $C_{20}(n-6)$ and $22:4(n-6)$. Similar changes were observed in the ab-

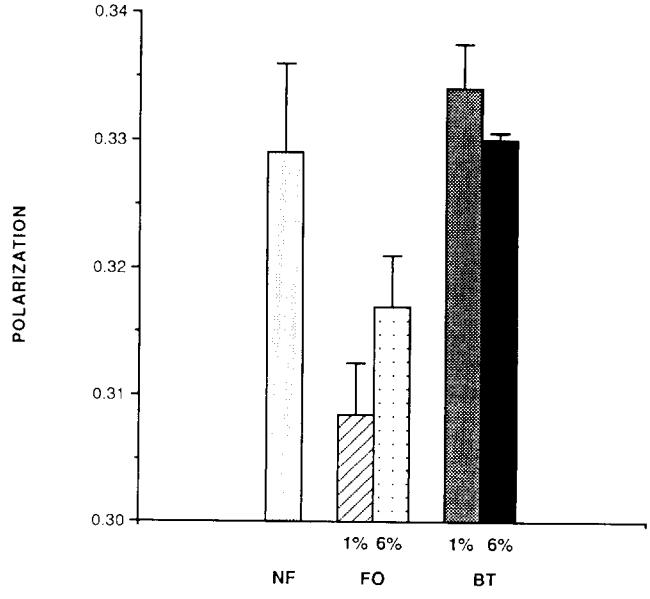

Fig. 3. Polarization fluorescence values of TMA-DPH incorporated in membranes of RDM4-L1 cells. NF, no fat; BT, beef tallow; FO, fish oil. Data represent the mean ± standard error of three experiments compared by Student's t-test. No significant difference was demonstrated between NF and BT 1% or BT 6%, as well as between FO 1% and FO 6%. However, the FO groups were significantly different from either the BT groups or the NF group ($P < 0.05$).

TABLE II

Relative amounts (mg%) of fatty acids of total lipid (TL) and phospholipid (PL) fractions of RDM4-L1 lymphoma cells grown in mice fed BT, FO or NF (no fat) diets

Values are means ± S.D. of four samples. The diets contained 1% fat by weight. Fatty acids are abbreviated in the usual manner: the first number indicates the number of carbon atoms and is followed by the number of double bonds; the number after $n-$ indicates the number of carbon atoms between the methyl end of the molecule and the first double bond on this side. tr, trace. n.d., not detected. * $P < 0.05$; ** $P < 0.01$ for corresponding fractions between BT and FO.

Fatty acid	BT		FO		NF	
	TL	PL	TL	PL	TL	PL
14:0	1.0±0.2	0.9±0.1	1.0±0.2	1.0±0.2	1.1±0.1	0.9±0.1
16:0	19.8±0.6	20.5±0.7	20.0±1.0	20.6±0.5	19.1±0.7	19.2±0.8
16:1($n-7$)	3.3±0.2	3.7±0.2	3.0±0.3	3.6±0.1	3.5±0.2	3.8±0.2
18:0	11.9±0.4	10.3±0.3	11.5±0.5	10.3±0.7	11.3±0.6	10.1±0.5
18:1($n-9$)	24.5±1.7	26.3±0.3	20.5±1.5 **	24.5±0.5 **	24.8±0.7	27.3±0.3
18:2($n-6$)	8.0±0.5	8.8±0.1	9.2±3.0	9.6±0.4 **	6.7±1.4	8.3±0.1
18:3($n-3$)	3.1±2.1	1.6±1.3	5.3±3.2	1.3±1.1	3.0±2.4	1.4±1.5
20:3($n-6$)	2.1±0.7	2.4±0.1	1.1±0.2 **	1.2±0.1 **	1.7±0.2	2.1±0.1
20:4($n-6$)	10.8±0.7	13.4±0.3	3.9±0.4 **	4.7±0.2 **	9.0±0.4	12.0±0.4
20:5($n-3$)	tr	n.d.	1.9±1.1 *	3.2±0.1 **	tr	tr
22:4($n-6$)	1.2±0.3	1.4±0.1	0.2±0.4 **	0.1±0.2 **	1.1±0.7	1.5±0.1
22:5($n-6$)	1.2±0.6	1.1±0.1	1.1±1.0	0.7±0.6	2.0±1.0	1.3±0.2
22:5($n-3$)	0.3±0.1	0.2±0.0	3.3±0.2 **	3.4±0.1 **	1.0±1.4	0.3±0.1
22:6($n-3$)	4.1±0.8	4.0±0.1	10.6±0.6 **	11.8±0.4 **	3.1±1.4	4.1±0.2
Others	2.8±0.7	2.7±0.5	1.8±0.1	1.2±0.2	2.9±0.7	3.2±0.7

solute amounts of these fatty acids, but not for 18:1($n-9$) and 18:2($n-6$) (data not shown).

Discussion

In this work, we have shown that dietary ($n-3$) polyunsaturated fatty acids had a profound effect not only on the lipid composition of the plasma membranes of tumor cells, but also on their membrane fluidity and the expression of the CD4 marker. Because diet-induced differences in cell plasma membrane phospholipids fatty composition is subject to exquisite homoeostatic control (a strain dependent effet) [3], it is difficult to predict how polyunsaturated fatty acids of the ($n-6$) series would influence the different parameters considered in this system. In a preliminary experiment, RDM4 cells from AKR donors fed 8% either FO ($n-3$) or safflower oil ($n-6$) exhibited similar membrane fluidization, as compared to donors fed BT (Bischoff, personal communication).

In the present case, membrane changes occured when fat represented as little as 1%, by weight, of the total food intake. When fats represented up to 16% of food intake, comparable results were observed regarding CD4 expression (not shown), in vivo tumor growth and relative amounts of membrane ($n-3$) and ($n-6$) polyunsaturated fatty acids [5]. Therefore, the differences we observed do not seem to be related to deficiency in dietary essential fatty acid but rather to the membrane fatty acid components. It is of particular interest to note that of all the membrane markers tested, only CD4 was modulated. More precisely, the percentage of CD4 positive lymphoma cells exhibiting sites associated with 'high' fluorescence intensity was the only one affected by diet, whereas the percentage of cells exhibiting 'low' fluorescence CD4 sites remained unaffected.

Since fluidization is a phenomenon which affects the entire lipid bilayer of the membrane and, ipso facto, all the membrane proteins which are anchored in it, the selectivity regarding CD4 modulation cannot be readily interpreted as a consequence of increased fluidity. Selective modulations of a membrane marker [8] or receptor [12] by changes of hydrogenation level or oxydative activity of membrane lipids have already been reported by others. Both abundance of unsaturated fatty acids and the number of double bonds could play a determining role in the expression of some receptors [12].

In this work, we observed CD4 modulation on 'wild' lymphoma cells as well as on lymphoma line selected for higher expression of this marker, but not in normal lymphoid organs. Should some heterogeneity have persisted in the RDM-4-L1 cells, the data on Fig. 1 could also be explained by a dietary fats driven shift in cell population, favoring those cells expressing the 'low' fluorescence intensity for CD4 sites. This could be an illustration of the fact that different lymphoid cells, upon stimulation, have different metabolic requirements in fatty acids [13].

The mechanism by which CD4 modulation occurred could be either peculiar to this tumor line or associated with the neoplasic transformation of these lymphoid

cells. Whether this action results from a down regulation of CD4 gene expression or from post-translation events remains to be established.

The latter concept is supported by the recently published finding that addition, to cultured lymphocytes, of certain gangliosides selectively modulate the expression of the CD4 marker [14]. It remains to be determined whether this phenomenon has an in vivo counterpart, but increasing evidence points to gangliosides, as well as other sphingolipids, as key participants in important physiologic functions [15]. As sphingolipids of the cell membrane can be affected by dietary fats [2], it cannot be excluded that dietary fats influence CD4 expression via an action on gangliosides which are thought to be mainly situated on the outer side of the plasma membrane, and to contribute to its biophysical properties [16]. Variation in ganglioside expression has recently been reported, between primary cell line and metastatic variants from the Lewis lung carcinoma [17], suggesting that ganglioside composition of tumor cell membrane may have a significant influence on the host-tumor relationship. Gangliosides have also been considered as important regulatory or structural molecule in the recognition of tumor cells by LAK cells [18], and the hypothesis that dietary fats influence such gangliosides is compatible with the finding that the RDM-4 lymphoma cells were found to be less sensitive to the cytolytic action of LAK cells, when harvested from FO-fed donors [5]. Experimental verification of this hypothesis is therefore the next step of this study.

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