ELSEVIER

Biochimica et Biophysica Acta 1236 (1995) 39–50

BBA Biochimica et Biophysica Acta

Docosahexaenoic acid-induced alteration of Thy-1 and CD8 expression on murine splenocytes

Laura J. Jenski [a,*], Gretchen M. Bowker [a,b], Michael A. Johnson [a], William D. Ehringer [a,1], Terry Fetterhoff [b], William Stillwell [a]

[a] *Department of Biology, Indiana University – Purdue University at Indianapolis, 723 W. Michigan Street, Indianapolis, IN 46202-5132, USA*
[b] *Boehringer-Mannheim Corporation, Indianapolis, IN 46265, USA*

Received 21 November 1994; accepted 18 January 1995

Abstract

Here we test whether the incorporation of docosahexaenoic acid (DHA, 22:6), an $(n-3)$ fatty acid, into lymphocyte membranes affects the expression of the surface proteins Thy-1.2 and CD8. DHA was incorporated into splenocytes by three methods: feeding mice diets containing menhaden (fish) oil, fusing splenocytes with DHA-containing phosphatidylcholine vesicles, and culturing splenocytes with DHA. Thy-1.2 and CD8 expression were measured by flow cytometry and complement-mediated lysis using a panel of monoclonal antibodies. As $(n-3)$ fatty acid incorporation into the lymphocytes increased, the expression of one Thy-1.2 epitope and one CD8 epitope decreased; the expression of two CD8 epitopes increased. Although diet-induced changes in surface protein expression may result from selective migration of cell populations or the diet's effect on protein biosynthesis, fusion with lipid vesicles demonstrated that DHA-containing phospholipids can mediate a direct and immediate effect. The decrease in Thy-1.2 expression was sustained for more than a week after removal of $(n-3)$ fatty acids from the diet, most likely due to retention of membrane-bound $(n-3)$ fatty acids. Because Thy-1.2 and CD8 participate in T cell activation, modulation of their expression by DHA suggests that DHA, when serving as a membrane structural element, may alter immune function.

Keywords: Docosahexaenoic acid; $(n-3)$ Fatty acid; Thy-1; CD8

1. Introduction

There is a growing interest in polyunsaturated fatty acids of the $(n-3)$ series, fatty acids in which the last double bond is three carbons from the methyl or omega end of the acyl chain. The $(n-3)$ fatty acid docosahexaenoic acid (DHA, 22:6) is the most unsaturated fatty acid found in any biological system, containing twenty two carbons and six unsaturations at positions 4, 7, 10, 13, 16 and 19. DHA is usually found in large amounts in brain, rod outer segment, and sperm [1], where it presumably serves a specific purpose; however, DHA may be increased in other tissues through diet.

Long chain $(n-3)$ fatty acids are abundant in fish oil, and associated with health benefits for neurologic development, cardiovascular disease, cancer, and arthritis. By influencing immunologic processes, fish oil-rich diets protect against autoimmune disease in strains of mice prone to systemic lupus erythematosus, and they lower susceptibility to rheumatoid arthritis in normal mice [2–4]. In part, $(n-3)$ fatty acids act by altering production of eicosanoid hormones (prostaglandins and leukotrienes) [5,6], thereby reducing inflammation. Leukotrienes may modulate T cell populations by inhibiting $CD4^+$ but enhancing $CD8^+$ T cell proliferation [6], and thus, not unexpectedly, dietary $(n-3)$ fatty acids are associated with increased $CD8^+$ [7] but not $CD4^+$ [8] T cell populations.

In addition to altering eicosanoid hormone production,

Abbreviations: 18:0,18:1 PC, 1-stearoyl,2-oleoylphosphatidylcholine; 18:0,22:6 PC, 1-stearoyl,2-docosahexaenoylphosphatidylcholine; BSA, bovine serum albumin; CO, corn oil; Con A, concanavalin A; DHA, docosahexaenoic acid; FBS, fetal bovine serum; GPI, glycosylphosphatidylinositol; HCO, hydrogenated coconut oil; MO, menhaden oil; PBS, phosphate-buffered saline; PTK, protein tyrosine kinase; RI, regression index.

* Corresponding author. E-mail: ljenski@indyvax.iupui.edu. Fax: +1 (317) 2742846.

[1] Current address: Department of Physiology and Biophysics, University of Louisville, Louisville, KY 40292, USA.

SSDI 0005-2736(95)00034-8

$(n-3)$ fatty acids play a structural role in biological membranes. Enrichment of membranes with DHA alters fluorescence of the membrane probe MC540 [9] and increases membrane permeability [10], structural alterations consistent with the induction of discreet membrane domains. If DHA-rich membrane domains form, then membrane proteins may segregate to some extent into domains having the preferred lipid environment. Protein function is known to be affected by the lipid environment [1]. The specific question yet to be answered is the following: if DHA alters lymphocyte plasma membrane structure and affects surface proteins, will DHA then modulate immune responses through a structural as well as a hormonal mechanism?

One lymphocyte membrane protein that is a candidate for modulation by DHA is Thy-1, a glycosylphosphatidylinositol (GPI)-linked glycoprotein present primarily on murine T cells but also expressed on neuronal cells, non-neuronal accessory cells, fibroblasts and dendritic epidermal cells [11]. Thy-1 exists as two allelic forms, Thy-1.1 and Thy-1.2, that differ by the single amino acid at position 89 [12]. It is used as a marker for T cells, but its specific function remains unclear [12]; it may play a role during development, cell recognition, signal transduction, and in the proliferation pathways of certain cell types [13]. CD8, on the other hand, is a heterodimeric membrane protein whose subunits each have a single transmembrane domain [14]. CD8 is present on a subset of T cells, binds to a monomorphic portion of MHC I, and is associated with a protein tyrosine kinase acting during T cell activation [15]. It is not known whether the presence of DHA in the plasma membrane of T cells changes the expression of proteins such as Thy-1 and CD8, which are involved in cell activation, and in turn alters immune responses. Here we monitor the expression of Thy-1.2 and CD8 as a function of DHA incorporation into splenocytes through diet, liposome fusion, and cell culture.

2. Materials and methods

2.1. Mice and diets

Weanling BALB/c male mice (Harlan Sprague Dawley, Indianapolis, IN) were fed modified AIN-76 pelleted diets (ICN Nutritional Biochemicals, Cleveland, OH) that contained 20% casein, 0.3% DL-methionine, 15% cornstarch, 5% alphacel, 0.2% choline bitartrate, 3.5% AIN-76 mineral mixture, 1% AIN-76 vitamin mixture, 44.5% sucrose and 0.5% corn oil (CO) plus an additional 10% oil as CO, hydrogenated coconut oil (HCO), or menhaden oil (MO, a generous gift from Zapata Haynie Corporation, Reedville, VA). The six diets are outlined in Table 1. Table 2 shows the fatty acid composition of Diet 1 (10% corn oil), Diet 2 (10% hydrogenated coconut oil), and Diet 6 (10% menhaden oil). The mice received tap water ad libitum and fresh food (approximately twice their usual daily consumption) at the beginning of the dark cycle (7 pm–7 am). Uneaten food was removed at the beginning at the light cycle. Mice were maintained on the diets for 3 weeks or the amount of time indicated. There was no significant difference between diets in mouse weight gain or the amount of food eaten. Mice that were not on experimental diets (adult BALB/c and ICR males, both strains from Harlan Sprague Dawley) received Purina rodent feed #5001 and tap water ad libitum. Mice were killed under the guidelines of the Panel on Euthanasia of the American Veterinary Medical Association. There were three to five mice per experimental diet group.

Table 1
Fat composition of experimental diets

Oil	% (w/w); diet designation					
	1	2	3	4	5	6
Corn	10.5	0.5	0.5	0.5	0.5	0.5
Hydrogenated coconut	0	10	7.5	5	2.5	0
Menhaden	0	0	2.5	5	7.5	10

2.2. Flow cytometry

Splenocytes were washed with phosphate buffered saline containing 0.1% sodium azide and 0.2% bovine serum albumin (PBS-NaN_3-BSA), and incubated in 100 μl of PBS-NaN_3-BSA plus 1 μg of phycoerythrin-conjugated anti-Thy-1.2 antibody (clone 30-H12, Boehringer-Mannheim, Indianapolis, IN) for 30 min on ice. The cells were then washed with PBS-NaN_3-BSA, and red blood cells were lysed by osmotic shock. Cell viability was determined with propidium iodide (Boehringer Mannheim, Indianapolis, IN). Fluorescence was measured on an Epics Profile II flow cytometer (Coulter, Hialeah, FL). The

Table 2
Fatty acid composition of diets 1, 2, and 6 [a]

Fatty acid	% of total fatty acids		
	corn oil diet 1	hydrogenated coconut oil diet 2	menhaden oil diet 6
14:0	0.1	22.6	7.8
14:1	0.0	1.3	0.0
16:0	13.2	19.1	14.0
16:1	0.0	0.0	10.0
18:0	2.3	17.0	1.8
18:1($n-9$)	31.0	10.0	13.7
18:2($n-6$)	51.7	21.0	6.8
18:3($n-3$)	1.1	0.3	3.3
20:1	0.1	0.0	1.2
20:2	0.0	1.3	0.1
20:4($n-6$)	0.0	0.0	0.5
20:5($n-3$)	0.0	0.0	13.4
22:5($n-3$)	0.0	0.1	1.7
22:6($n-3$)	0.0	0.0	9.7

[a] Fatty acids found at less than 0.5% are not shown.

lymphocyte population was gated using forward scatter and side scatter, and a phycoerythrin-conjugated rat anti-DNP antibody was used as a negative control. Each cell population was stained in triplicate and all samples were run once each.

2.3. Flow cytometry data analysis

Histograms were compared by linear regression analysis with the program Regress® (Boehringer-Mannheim, Indianapolis, IN) [16]. Fluorescence values for each channel of two histograms were treated as paired data (x, y); identical histograms produce a straight diagonal line. Residuals (difference between the observed values versus values expected for identical histograms) were calculated for each point, and the mean residual was expressed as a percentage of the peak height of one histogram. This value, the regression index (RI), is positively related to the disparity between histograms, and zero for identical histograms.

2.4. Preparation of ^{51}Cr-labeled Con A blasts

Splenocytes from two mice were cultured in 60 mm tissue culture dishes at $5 \cdot 10^6$ cells/ml of RPMI 1640 plus antibiotics, 25 mM Hepes buffer, 2 mM glutamine, 50 μM 2-mercaptoethanol, 5% fetal bovine serum (FBS), and 2.5 μg of concanavalin A (Con A, Pharmacia Biotech, Piscataway, NJ) per ml (5–7 ml/dish). After 2 days in a humidified 37° C atmosphere with 5% CO_2, the cells were harvested, washed in PBS, and labeled for 1 h in 100–150 μCi sodium [^{51}Cr]chromate (New England Nuclear-Dupont, Boston, MA) in < 1 ml of RPMI plus 5% FBS. Washed, labeled blasts were suspended at $1 \cdot 10^5$ cells/ml of RPMI plus 20% FBS (either heat inactivated or untreated).

2.5. Preparation of fatty acid-supplemented medium

Our method is based on that of Spector and Hoak [17]. Briefly, 5 mg of free fatty acid, 22:6($n-3$) (NuChek, Elysian, MN), was dissolved in 5 ml of hexane, and then added to a thin layer of Celite powder (1 μmol fatty acid/1 g Celite; Sigma). After evaporating the hexane under nitrogen, the fatty acid-Celite mixture was added to 100 ml of 1% (w/v) BSA in RPMI 1640 (supplemented with antibiotics, 25 mM Hepes buffer, and 2 mM glutamine). The suspension was stirred for 30 min at room temperature in the dark, and the supernatant was recovered by centrifugation at $11\,000 \times g_{max}$ for 10 min at 4° C, followed by filtration through a 0.22 μ Millipore disposable filter unit. Control medium was prepared similarly, but without the addition of free fatty acid. The media were stored frozen until used, at which time they were supplemented with FBS and 2-mercaptoethanol. A preliminary experiment in which 5 mg of 22:6 was spiked with 0.5 μCi (1.5 ng) of [^{3}H]20:4 indicated that more than 50% of the fatty acid (i.e., 25 μg/ml) eluted into the BSA solution (Stone, J. and Jenski, L., unpublished observation).

2.6. Cell lysis with antibody and complement

^{51}Cr-labeled splenic Con A blasts were distributed into replicate wells ($1 \cdot 10^4$ cells in 100 μl/well) of microtiter plates to which 100 μl of monoclonal antibody had been added; in each assay, four dilutions of each antibody were tested. Monoclonal anti-Thy-1 clone 30-H12 (rat IgG2b, FITC-conjugated) was obtained from Becton Dickinson (Mountain View, CA). The anti-CD8 antibodies were conditioned media from cell lines HO-2.2 (anti-Lyt-2.2, mouse IgM) [18], 3.155 (anti-Lyt-2, rat IgM) [19], and 2.43 (anti-Lyt-2.2, rat IgG2b) [19] purchased from the American Type Culture Collection (ATCC, Rockville, MD). The plates were incubated for 30 min at 4° C, washed three times in PBS plus 20% FBS, and then incubated for 30 to 60 min at 37° C with complement (Low Tox M rabbit complement, Cedarlane, Hornby, Ontario, Canada), diluted 1:20 to 1:40. Wells that lacked antibody received either complement (background), medium only (spontaneous release), or detergent (maximum release). After centrifugation ($400 \times g$, 5 min), 100 μl was removed from each well, and radioactivity present in the supernatant was measured in a Beckman Gamma 5500 gamma counter (Fullerton, CA). Percent specific lysis was calculated as ((experimental cpm − background cpm)/(maximum cpm − background cpm)) × 100. Significant differences ($P \leq 0.05$) were determined by an independent Student's t-test with the program WinStar (Anderson Bell, Arvada, CO).

2.7. Lipid vesicle-cell fusions

The phospholipids 1-stearoyl,2-oleoylphosphatidylcholine (18:0,18:1 PC) and 1-stearoyl,2-docosahexaenoylphosphatidylcholine (18:0,22:6 PC) (Avanti Polar Lipids, Alabaster, AL) were dissolved in chloroform and dried under a stream of nitrogen followed by overnight vacuum. Lipids were hydrated in PBS and sonicated on ice for 5 min with a Heat Systems W-380 cell disrupter at setting 5 to produce small unilamellar vesicles. Lipid vesicle suspensions (0.125 to 1.0 mg/ml of PBS) were mixed with splenocytes (10^8 cells/ml vesicle suspension) at room temperature for 1 h in the dark. The cells were then washed once with cold PBS + 10% BSA, and three times with PBS + 10% FBS. In some experiments, 18:0,22:6 PC was spiked with the fluorescent lipid N-(lissamine rhodamine B sulfonyl)diacylphosphatidylethanolamine (Avanti Polar Lipids, Alabaster, AL) at a molar ratio of 300:1 to monitor the efficiency of lipid fusion by fluorimetry (MPF-66, Perkin-Elmer, Norwalk, CT).

2.8. Gas chromatography

Cells were extracted with $CHCl_3/CH_3OH$ (2:1, v/v), and the lipid extracts were rotovaporated to a small vol-

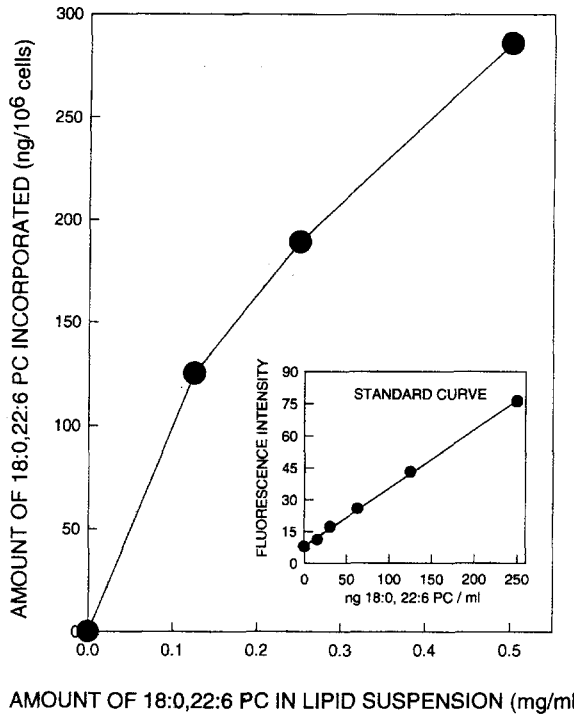

Fig. 1. Estimation of lipid vesicle fusion with lymphocytes. A trace amount of fluorescent phosphatidylethanolamine was added to 18:0,22:6 PC, which was fused at various concentrations with splenic blasts. The amount of 18:0,22:6 PC incorporated into cells was estimated from cell-associated fluorescence; the standard curve from which this information was derived is shown in the inset. The 'zero' lipid control lacked the fluorescent tracer.

ume. Lipids were saponified in 0.5 M methanolic NaOH by refluxing for 1 h. Fatty acids were esterified by the addition of BF_3-methanol and refluxing again for an additional 30 to 45 min. Methyl esters were extracted in hexane and rotovaporated to a small volume. Two gas chromatography systems were used: 2 μl of the methyl esters were analyzed on a SRI Gas Chromatograph (Torrance, CA) equipped with a flame ionization detector and a packed column of 10% SP-2330 on 100/120 Chromosorb WAW. The oven temperature increased from 190° C to 225° C over 25 min during the course of the run. Alternatively, 1 μl of the fatty acid methyl esters was injected into a Shimadzu GC-17A gas chromatograph equipped with a Stabilwax capillary column (0.25 mm × 30 m; Restek, Bellefonte, PA), and the following temperature ramp was used: 180° C for 1 min, increased at 5° C per min to 245° C, held at 245° C for 10 min. Identification of the peaks was performed by direct comparison to the retention times of methyl ester standards (NuCheck, Elysian, MN).

3. Results

3.1. Effect of DHA on Thy-1 expression

Both our own work [20,21] and that of others [8,22–24] suggest that changes in membrane fatty acid composition

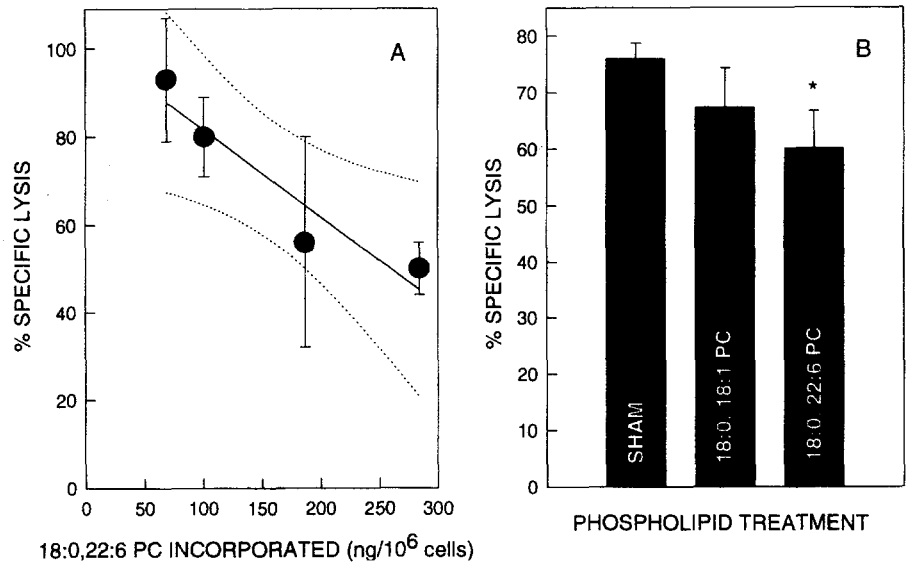

Fig. 2. Lymphocytes enriched with DHA by liposome fusion show an apparent decrease in Thy-1 expression. (A) ^{51}Cr-labeled splenic blasts were modified with various amounts of 18:0,22:6 PC, and then treated with anti-Thy-1.2 plus complement. Each point is the mean ± standard deviation ($n = 3$). The solid line is a first order linear regression; the dotted lines are the 95% confidence limits. The data suggest a significant correlation between 18:0,22:6 PC and specific lysis ($P = 0.0516$, Pearson product moment correlation). (B) ^{51}Cr-labeled splenic blasts were treated with either 18:0,18:1 PC or 18:0,22:6 PC (initial concentrations adjusted to give equimolar incorporation), or left untreated (sham), and then assayed for Thy-1 expression with anti-Thy-1.2 plus complement. The data shown are means ± S.D. ($n = 3$); the asterisk demonstrates significant difference from the sham-treated control ($P < 0.05$, Student's t-test).

may affect surface proteins. Thus, we predicted that the presence of DHA in the splenocyte membrane may alter the apparent expression of T cell surface molecules. To test the direct effect of a DHA-containing phospholipid on Thy-1 expression, we fused 18:0,22:6 PC lipid vesicles with splenic Con A blasts in vitro. In some experiments, fusion was monitored with a fluorescent phospholipid included in the 18:0,22:6 PC vesicles. As shown in Fig. 1, the amount of fluorescent lipid incorporated into cells was proportional to the concentration of the lipid suspension used in the fusion, and the amount of 18:0,22:6 PC associated with the cells could be estimated from the fluorescence intensity standard curve. We demonstrated previously that efficiency of fusion correlated with the

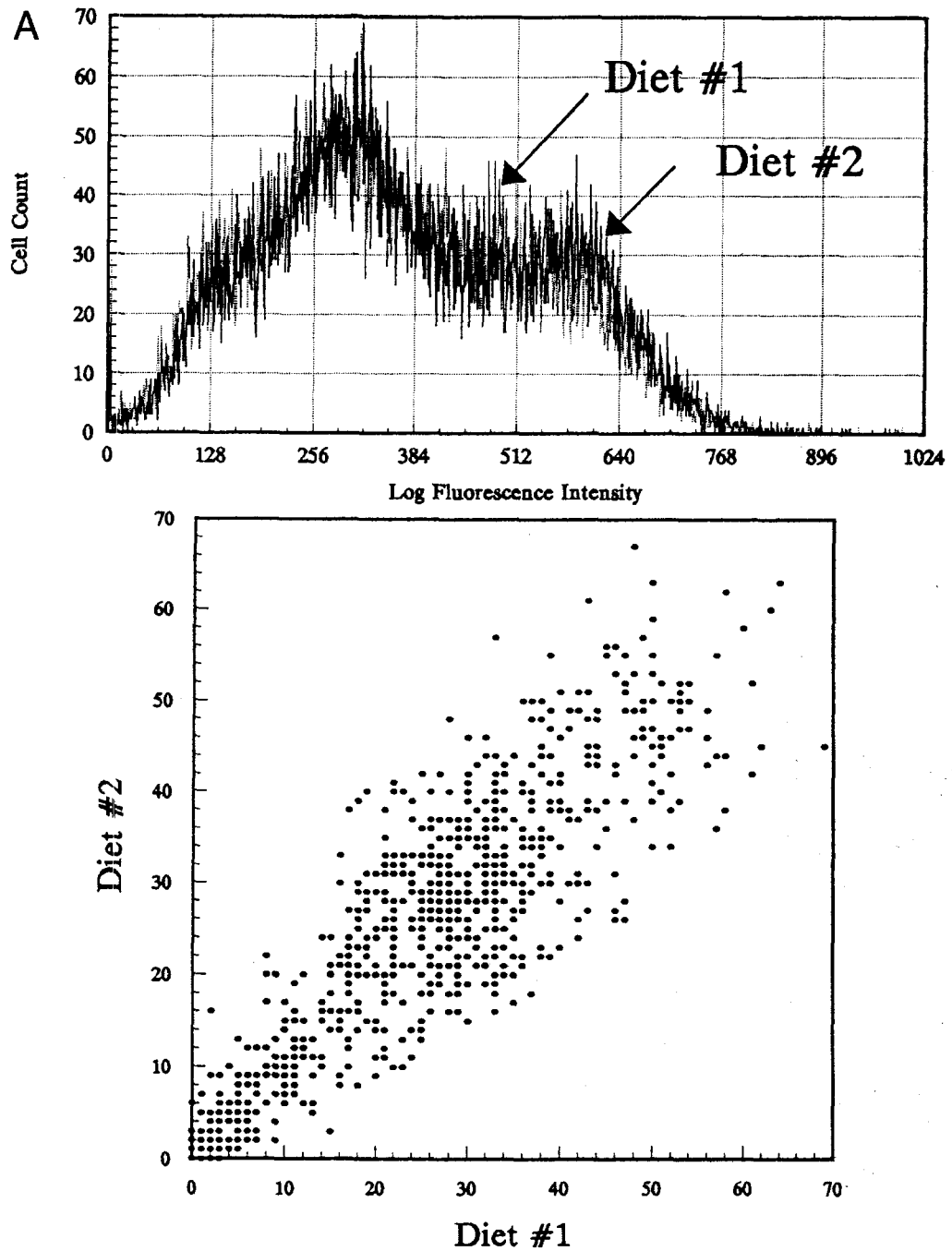

Fig. 3. Dietary MO is associated with decreased expression of Thy-1. Weanling mice were fed diets containing 10.5% CO (diet 1), or 0.5% CO plus 10% HCO (diet 2) or MO (diet 6) for 3 weeks. Splenocytes from three mice fed identical diets were pooled, stained with phycoerythrin-conjugated anti-Thy-1.2, and analyzed by flow cytometry. (Upper panel) Overlaid histograms of splenocytes from CO (solid line) and HCO-fed (dotted line) mice, and the corresponding regression analysis. (Lower panel) Overlaid histograms of splenocytes from CO (solid line) and MO-fed (dotted line) mice, and the corresponding regression analysis.

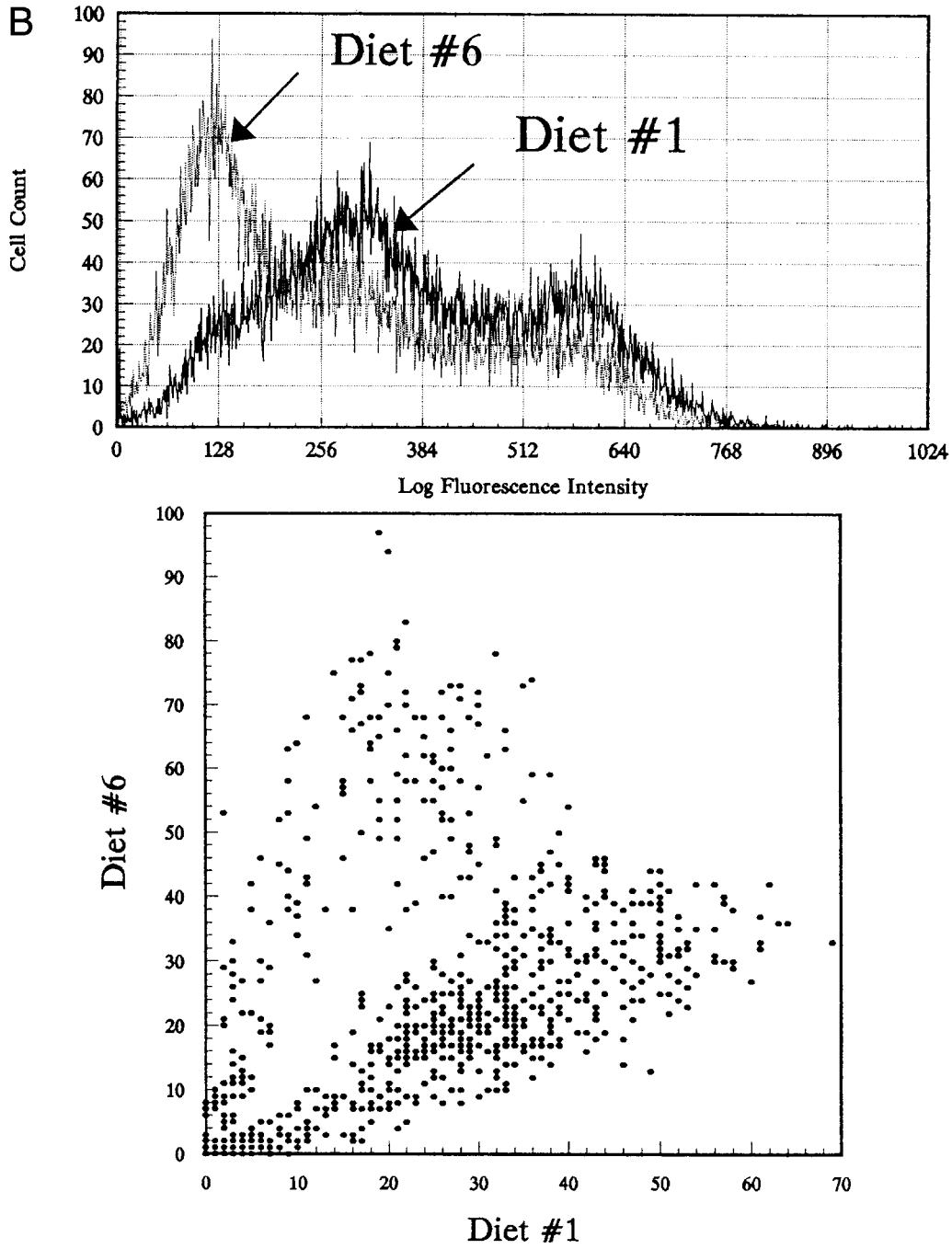

Fig. 3 (continued).

type of carrier lipid [21], indicating that the fluorescent probe was not entering the cells independently of the carrier phospholipid.

To measure Thy-1 expression, we used anti-Thy-1.2 in a complement-mediated lysis assay. In this assay, cell lysis (^{51}Cr release) is dependent upon the binding of antibodies to cell surface molecules (e.g., Thy-1) followed by the activation of complement to the terminal, pore-forming stage. Fig. 2A demonstrates that fusion of 18:0,22:6 PC with Con A-stimulated spleen cell blasts reduced Thy-1 expression (measured by complement-mediated lysis) in a dose-dependent fashion. Fusion with 18:0,22:6 PC modulated Thy-1 expression more dramatically than 18:0,18:1 PC (Fig. 2B); the phospholipid concentrations of the vesicle suspensions were adjusted to achieve equimolar phospholipid incorporation. We previously reported the behavior of 18:0,18:1 PC to be intermediate between 18:0,22:6 PC and sham treatments in a similar system [21]. Thy-1 expression was monitored in a total of four fusion experiments.

In these experiments we have introduced DHA into the plasma membrane in PC molecules. PC is the predominant

Table 3
Thy-1 expression on splenocytes stimulated with Con A in culture medium supplemented with DHA

Medium supplement	% Specific lysis [a] (percent of control)	
	experiment 1	experiment 2
None	57.1 ± 4.4	67.6 ± 0.7
22:6($n-3$)	48.8 ± 2.0 [b] (85%)	59.1 ± 4.1 [b] (87%)

[a] Means ± S.D. ($n = 3$).
[b] Significantly different from control (no fatty acid supplement), $P < 0.05$, Student's t-test.

phospholipid in the outer leaflet of most biological membranes, and DHA can be found in PC. However, lymphocytes may not naturally produce a plasma membrane with DHA exclusively in PC at these levels. Therefore, we tested whether splenocytes, after incorporating DHA into membrane phospholipids via their own metabolic pathways, also showed decreased Thy-1 expression. We cultured splenocytes with medium containing albumin-bound DHA (and Con A) for 48 h. Relative Thy-1 expression was then quantified by a ^{51}Cr release assay with anti-Thy-1.2 and complement. The two independent experiments performed are shown in Table 3. In each case, there is a significant, albeit small decrease in Thy-1 expression on splenocytes cultured with DHA compared to control medium ($P < 0.05$, Student's t-test). Fatty acid was incorporated from the medium into the cells, causing the percent of DHA among total fatty acids to increase more than three fold (Table 4). Thus, it is likely that DHA is entering triacylglycerol as well as phospholipid pools, and internal as well as plasma membranes. However, whether introduced as a preformed phospholipid or free fatty acid, DHA is associated with reduced expression of this Thy-1 epitope.

In biological systems, DHA is efficiently incorporated from the diet into cellular membranes. Hence, we predicted that Thy-1 expression on splenocytes would decrease if mice were fed a fish oil-containing ($(n-3)$ fatty acid-rich) diet. In our experiments, weanling BALB/c male mice were fed diets containing essential fatty acids and 10% additional fat as some ratio of HCO and MO, or as CO. After 3 weeks on the diets, the spleens were removed, and single cell suspensions were stained with phycoerythrin-labeled anti-Thy-1.2 and analyzed by flow cytometry to produce the histograms shown in Fig. 3.

Table 4
Gas chromatography of splenocytes stimulated with Con A in culture medium supplemented with DHA

Fatty acid [a]	% of total fatty acids; fatty acid supplement in medium	
	control	22:6
14:0	2.2	2.9
16:0	34.0	36.2
16:1	6.0	0.8
18:0	16.6	15.3
18:1($n-9$)	14.0	7.3
18:2($n-6$)	4.9	3.4
20:4($n-6$)	10.4	7.1
22:4($n-6$)	0.9	0.4
22:5($n-3$)	1.3	0.6
22:6($n-3$)	6.5	22.6

[a] Only fatty acids found at greater than 0.5% are shown.

Histogram similarity was assessed by linear regression using the program Regress® (Boehringer-Mannheim, Indianapolis, IN) [16]. Histograms generated using anti-Thy-1.2-stained cells from mice on different diets were overlaid and the number of cells per fluorescence intensity treated as paired data. For similar diets, there would be no difference in the resultant histograms, and the paired data would yield a significant correlation with a slope of 1.00 (line of identity). As histograms become less similar, the paired data would begin to deviate from the line of identity. This deviation per data point represents the residual values. The mean residual is expressed as a percentage of the control histogram peak height (because the units are cell numbers rather than fluorescence intensities) to yield a regression index (RI). Fig. 3A shows regression analysis that resulted in similar histograms; these were histograms of Thy-1 expression on splenocytes from mice fed corn oil and hydrogenated coconut oil-rich diets. The paired data show a good correlation about the line of identity with a resultant RI of 8.11. Fig. 3B demonstrates visibly dissimilar histograms, data from corn oil and menhaden oil-fed groups. The paired data show correlation about a line (slope < 1.0) with positive residuals in the first 200 channels resulting in an RI of 31.50. The regression index therefore represents a sensitive index for monitoring gradual changes in cytometric histograms that may not be detected using mean fluorescence intensity alone [16].

The data in Fig. 3, a representative experiment of the three performed, demonstrate that splenocytes from saturated fat (HCO)-fed mice displayed Thy-1 expression equivalent to CO-fed (control) mice, whereas MO-fed splenocytes showed a decrease in the percentage of brightly-staining cells and an increase in the percentage of negative cells. It was not clear whether the alteration was the result of brightly staining cells becoming negative, or a general decrease in Thy-1 expression in which the bright cells became dull, and the dull cells became negative. In either case, however, this analysis is consistent with the results of the complement-mediated lysis assay. Propidium iodide staining was also done to ensure viability, and the six diet groups were more than 98% viable. The histogram for diet 1 (10.5% CO) was overlaid sequentially with those of the other five diets to generate RI values. The greater the RI value, the more disparate the two histograms. As shown in Fig. 4, the RI increased as the percentage of MO in the diet (diet number) increased. This suggested that the apparent loss of Thy-1^{bright} cells from the spleen was associated with the presence of MO in the diet. Gas

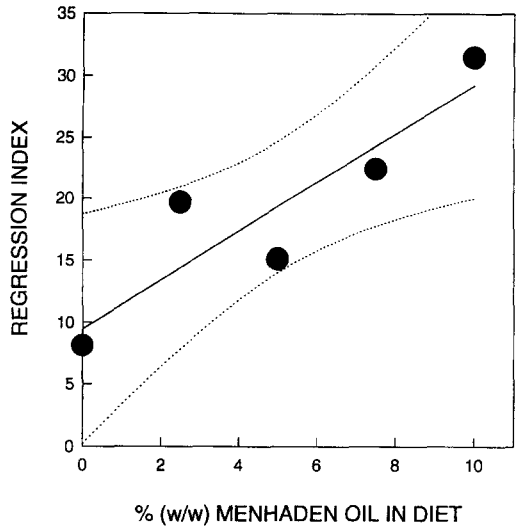

Fig. 4. Increased dietary MO correlated with decreased Thy-1 expression. Weanling mice were fed various amounts of CO, HCO, and MO (diets 1 to 6) for 3 weeks. Splenocytes from three mice on identical diets were pooled, stained with phycoerthrin-conjugated anti-Thy-1.2, and analyzed by flow cytometry. Histograms from diets 2 through 6 were overlaid with the histogram from diet 1, and regression indices (RI) were calculated. The greater the RI value, the more Thy-1 expression deviates from diet 1. The solid line is a first order linear regression; the dotted lines are 95% confidence limits. There is a significant correlation between dietary MO and Thy-1 expression, i.e., RI ($P = 0.0366$, Pearson product moment correlation).

chromatography revealed an increase in DHA in splenocytes from MO-fed mice (Table 5).

Although the physiological conditions created by the MO diet may have fostered the migration of Thy-1^{bright} cells away from the spleen (or the migration of Thy-1^{dull} cells to the spleen), the experiments performed in vitro rule out selective migration as the sole mechanism for dietary fish oil's effect. To confirm that the two assays (flow cytometry and specific lysis) measured parallel events, we fused splenocytes with various amounts of 18:0,22:6 PC, stained them with phycoerythrin-conjugated anti-Thy-1.2, and analyzed them with the flow cytometer. As shown in Table 6, supplementation of splenocytes with 18:0,22:6 PC produced high RI values, and inspection of the histograms (not shown) revealed that the Thy-1^{bright} population was reduced in the 18:0,22:6 PC-treated groups. Reduced Thy-1 expression was most striking with unfixed cells, but also found in two additional experiments in which the cells were fixed prior to flow cytometry. The fused unfixed cells had greater than 95% viability as shown by propidium iodide staining. Gas chromatography revealed a modest increase in DHA due to the fusion of 18:0,22:6 PC with the splenocyte membrane. Thus, in a static lymphocyte population, immediately after the addition of DHA to the plasma membrane, the binding of anti-Thy-1.2 to the cells was reduced. This finding is consistent with the hypothesis that membrane-bound DHA alters some aspect of membrane protein structure.

Table 5
Fatty acid composition of spleen cells from mice fed experimental diets

Fatty acid	% of total fatty acids [a]				
Diet:	1	2	3	4	6
14:0,14:1	1.9	3.1	2.3	2.8	2.2
16:0	28.3	27.9	19.3	25.9	26.1
18:0	18.2	12.7	11.9	13.3	14.2
18:1($n-9$)	21.3	23.9	17.0	19.4	18.3
18:2($n-6$)	15.5	5.0	7.0	7.8	6.6
18:3($n-3$)	–	6.3	1.8	tr	2.2
20:4($n-6$)	4.4	8.2	2.1	3.5	3.6
20:5($n-3$)	–	–	3.2	4.8	5.3
22:4($n-6$)	5.7	3.9	1.7	–	0.7
22:5($n-3$)	–	–	1.2	2.6	2.4
22:6($n-3$) [b]	–	–	2.7	5.8	6.3

[a] tr, trace; –, not detectable.
[b] Correlation between % dietary MO and % DHA, $P = 0.0238$ (Pearson product moment correlation).

Table 6
Thy-1 expression on splenocytes modified by fusion with 18:0,22:6 PC

Lipid concentration (mg/ml)	Relative Thy-1 expression (RI)	DHA content (% of total fatty acids)
0	0	0.2
0.25	10.55	1.4
0.5	12.08	0.7
1.0	15.17	2.1

3.2. Effect of DHA on CD8 expression

Thy-1 is implicated in T cell activation; however, during an immune response T cells are activated by recognition of foreign antigen. CD8 plays an accessory role in T activation by antigen, and thus is an interesting model for further study of DHA's effect on immunologic surface molecules. We supplemented spleen cells with DHA either by fusion with 18:0,22:6 PC or by culture in fatty acid-supplemented medium (plus Con A). We then used a panel of monoclonal antibodies against CD8 in the complement-mediated lysis assay to monitor CD8 expression.

As shown in Fig. 5 (one of four experiments), different epitopes on CD8 (α chain) were modulated by 18:0,22:6 PC. The epitopes bound by monoclonal antibodies HO-2.2 and 3.155 demonstrated a significant increase in expression, whereas the epitope detected by monoclonal antibody 2.43 showed a significant decrease. Besides indicating that CD8, as well as Thy-1, is sensitive to the membrane alterations associated with DHA, these results also emphasize that DHA's effect is more than a nonspecific decrease in antibody binding.

As before, we extended the experiments to measure epitope expression on Con A-stimulated splenocytes cul-

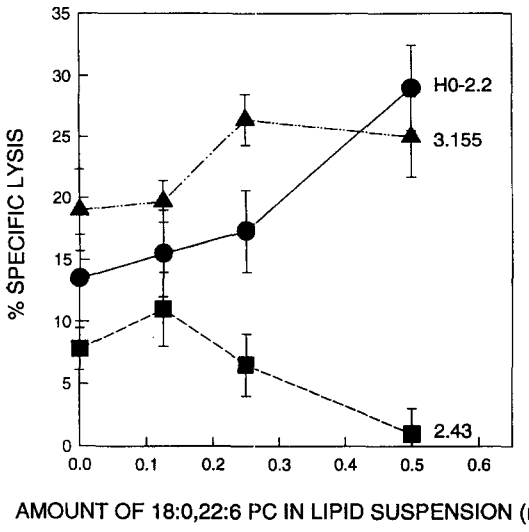

Fig. 5. Fusion of 18:0, 22:6 PC with splenocytes alters expression of three CD8 epitopes. ^{51}Cr-labeled splenic blasts were fused with various amounts of 18:0, 22:6 PC, and then treated with monoclonal anti-CD8 (HO-2.2, 2.43, and 3.155) plus complement. The data shown are means ± S.D. ($n = 3$).

tured in fatty acid-supplemented media. Fig. 6 shows that the incorporation of DHA into splenocytes increased binding of the HO-2.2 antibody and decreased the binding of the 2.43 antibody. In this experiment, the increase in 3.155 binding to DHA-supplemented cells did not reach statistical significance at the 95% confidence level ($P = 0.0695$, Student's t-test), although small but significant increases were found in other experiments. Importantly, the effects

Table 7
Comparison of lipid vesicle fusion and fatty acid-supplemented medium for their effects on CD8 expression

Lymphocyte treatment	% of no DHA control; CD8 epitope expression		
	HO-2.2	2.43	3.155
Fusion with 18:0,22:6 PC	215	13	132
Culture in DHA	181	50	112

of biosynthetic incorporation of DHA on CD8 expression match the effects of 18:0,22:6 PC (Table 7), both qualitatively and quantitatively.

3.3. Persistence of the fish oil diet effect

($n - 3$) fatty acids are preferentially retained in some cell types even after removal from the diet [1]. To test whether the influence of MO diet on Thy-1 expression persisted after removal of MO from the diet, we performed the following experiment. Mice were fed a diet containing 10% MO or 10% HCO for 3 weeks, and then half of the mice on the MO-containing diet were switched to a diet with 10% HCO. Periodically thereafter, lymphocytes were stained with anti-Thy-1.2, and the flow cytometry data were analyzed with the Regress® program. Nine days after the diet switch, comparison of MO-fed and diet-switched splenocytes produced an RI of 5.7, considerably lower than the RIs for comparison of MO and HCO-fed cells (RI = 19.5), and HCO-fed and diet-switched cells (RI =

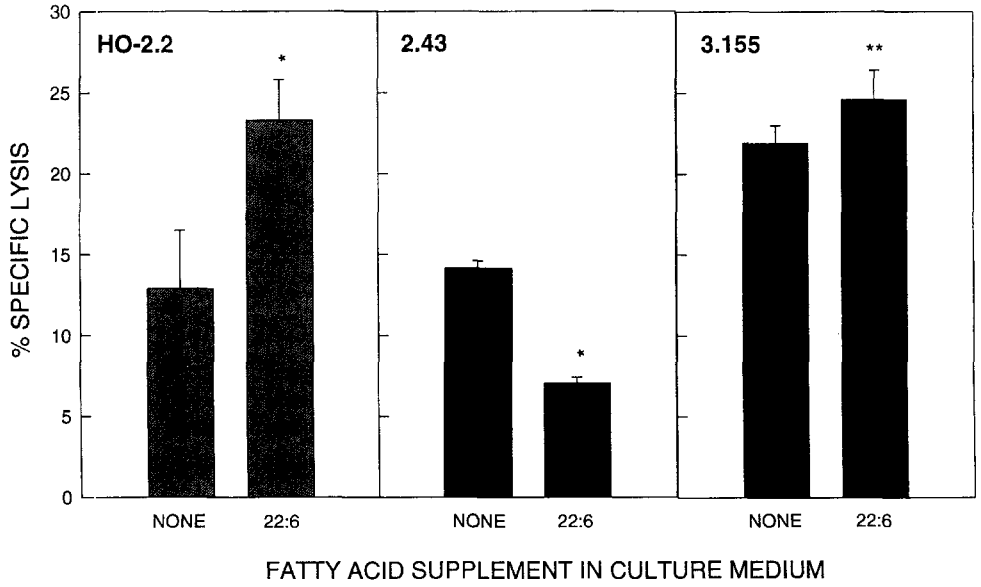

Fig. 6. Splenocyte culture in DHA-supplemented medium alters CD8 expression. Splenocytes were cultured in medium plus 5% FBS and ~ 25 μg/ml of BSA-bound 22:6 for 2 days in the presence of Con A. Splenic blasts were ^{51}Cr-labeled, and treated with monoclonal anti-CD8 (HO-2.2, 2.43, and 3.155) plus complement. The data shown are means ± S.D. ($n = 3$). One asterisk denotes significant difference from control ($P < 0.05$, Student's t-test); two asterisks denotes $P = 0.0695$.

20.6). This suggests that after 9 days on the HCO diet, splenocytes retained the phenotype characteristic of the MO diet. Tests made at later times, 21 and 35 days after switching the diets, produced high RIs (14 to 42) for the various comparisons. Thus, there was some retention of the diet-induced effect on Thy-1 expression, although in this experiment a long-lasting effect was not demonstrated.

4. Discussion

In this paper we demonstrate that the presence of DHA in lymphocyte plasma membranes is associated with decreased expression of Thy-1 (the epitope defined by monoclonal antibody from clone 30-H12) and altered expression of three CD8 epitopes (defined by monoclonal antibodies HO-2.2, 2.43, and 3.155). DHA was incorporated into membranes through diet (dietary fish oil), fatty acid-supplemented culture medium, and fusion with lipid vesicles of 18:0,22:6 PC. Although diet and cell culture may change cellular constituents other than the DHA content of membrane phospholipids, fusion occurs rapidly without opportunity for substantial metabolic changes. Additionally, although DHA may be incorporated into internal membranes through diet or culture, 18:0, 22:6 PC incubation with lymphocytes for a brief period in the cold is most likely to place DHA in the plasma membrane. Therefore, it is our opinion that the effect of DHA is related to its structural role in plasma membranes. Because both Thy-1 and CD8 are implicated in T cell activation, the changes in membrane lipid bilayer structure that influence protein structure (i.e., epitope expression) may influence Thy-1 and CD8 function during cell activation.

Earlier reports have provided evidence that Thy-1 is sensitive to the lipid components of the plasma membrane. Muller et al. [25] reported that splenocyte treatment with a phosphatidylcholine preparation ('active lipid') not only decreased lipid packing (as probed with DPH) but also reduced Thy-1 expression (as monitored by flow cytometry with anti-Thy-1.2, clone 30-H12). We have found a similar relationship between packing of 18:0, 22:6 PC (deduced from pressure/area isotherms) [9] and reduced Thy-1 expression (this paper), which is consistent with the work of Muller and coworkers. Muller et al. [25] also observed that cholesteryl hemisuccinate did not alter Thy-1 expression, although MHC I expression was reduced by cholesterol treatment. However, Traill and Wick [26] did describe work by these investigators in which cholesterol affected Thy-1 expression on the T cell lymphoma EL4. Twisk et al. [27] found by flow cytometry that lymphocytes from mice fed a 5% lard diet (containing principally 18:1, 16:0 and 18:0) had greater Thy-1 expression compared to a 5% sunflower diet (principally 18:2 and 18:1). Yet, in an earlier report, Twisk et al. [28] found no effect of culture in 18:2, 18:3, 20:4 or 20:0 on lymphocyte Thy-1 expression. Thus, Thy-1 expression is sensitive to membrane lipid composition, but the relationship between Thy-1 expression and membrane structure is complicated and poorly understood.

CD8 is less well studied with respect to fatty acid or lipid modulation of expression. Fernandes [7] reported increased CD8 expression on splenocytes from mice fed 20% fish oil compared to those fed 20% lard or corn oil; calorie restriction, associated with enhanced longevity, similarly increased the percentage of $CD8^+$ splenocytes. Oth et al. [8], in contrast, found no difference in CD8 (or Thy-1.1) expression on T cell lymphoma cells grown in fish oil and beef tallow-fed mice, whereas the fish oil diet was associated with lower CD4 expression. Direct comparison should be made carefully, however, because there are substantial differences between these two studies, and our study as well: cell type, monoclonal antibody clone, mouse strain (autoimmune-prone, leukemia-prone, and normal), oil sources (although the fatty acid compositions were comparable for the three fish oils, and for the three saturated fats), final fat content of the diets, and pre-existing DHA in membranes.

It is not clear what membrane change is reducing the binding of Thy-1 and CD8 to the monoclonal antibodies. There are three nonexclusive mechanisms by which DHA, esterified to membrane phospholipids, may affect membrane protein expression. These three mechanisms are conformational change, vertical displacement, and qualitative changes in neighboring proteins, all of which may result from DHA-induced membrane domain formation. The latter two mechanisms also incorporate the idea of epitope masking, i.e., neighboring proteins reducing accessibility of Thy-1 or CD8 epitopes to the antibody probes.

The underlying hypothesis is that DHA promotes the formation of membrane microdomains, those that are rich in DHA and poor in cholesterol, and those that are DHA-poor and cholesterol-rich. Cholesterol does not interact well with DHA, and thus is thought to be preferentially excluded from DHA-rich domains [9]. Because both DHA (as we have shown here) and cholesterol [26] influence Thy-1 expression, we predict that formation of DHA-rich/cholesterol-poor and DHA-poor/cholesterol-rich domains (by addition of DHA or cholesterol) will impel Thy-1 and CD8 to segregate into domains having their favored lipid environments. Segregation into domains may alter protein expression without necessitating a change in biosynthesis. Fatty acids are components of the annular lipids surrounding membrane proteins, and therefore DHA may affect protein conformation through this interaction. Additionally, because of its six double bonds, DHA is proposed to assume a helical structure and produce a bilayer that is thinner than saturated bilayers [29]. From the protein's perspective, this may change its position relative to other membrane components and the external milieu (e.g., antibodies). Furthermore, Thy-1 is GPI-linked, and DHA may be esterified to Thy-1's lipid anchor. Although

DHA is not prominent among fatty acid founds in PI, the DHA content of PI can be enriched through diet or cell culture [30,31]. The acyl chain composition of Thy-1 is reportedly variable [32], containing, in addition to stearic acid (18:0), alleged C20 and C22 fatty acids [33]. Thus, it is certainly of interest to test whether DHA may be covalently attached to Thy-1.

DHA-induced microdomains may also harbor a qualitatively or quantitatively different assortment of proteins, providing Thy-1 and CD8 with new neighbors that may mask epitopes, affect protein-protein interactions, and influence protein function. In the T cell plasma membrane, Thy-1 associates with another GPI-linked protein, Ly-6, in a large complex that also includes tyrosine kinases ($p56^{lck}$, $p60^{fyn}$, $p53/p56^{lyn}$) [34,35]; presumably the proximity of these molecules will affect the probability of their colliding and forming a complex. Because Thy-1 appears to diffuse in a domain 1 to 2 μm wide [36], and other GPI-linked proteins have a similar barrier-free path [37], one expects that the two molecules, Thy-1 and Ly-6, may hunt for each other and for kinases prior to complex formation. On the other hand, if Thy-1 naturally clusters in a microdomain of a given lipid composition, then it is likely to closely interact with those proteins gathered into the same domain. There is evidence to support lipid-induced clustering of GPI-linked proteins into membrane patches [38]. The testable hypothesis is that Thy-1 and Ly-6 prefer the same lipid environment.

Like Thy-1, CD8 associates with molecules on the cell surface, the T cell antigen receptor and a tyrosine kinase [15], and thus may cluster with its protein partners in microdomains. Structural changes induced by DHA may potentiate or curb cell activation, i.e., group or separate molecules. Although potentiation would, theoretically, heighten a T cell response, it may be equally advantageous to increase the threshold required for T cell activation to decrease the chance of autoimmunity. Clearly, $(n-3)$ fatty acids have hormonal effects that moderate autoimmune inflammation. However, in addition, the correlation between dietary $(n-3)$ fatty acids and reduced severity of autoimmune disease is consistent with DHA-induced membrane alterations that modulate T cell activation. It is now important to explore the relationship between DHA-induced changes in Thy-1 (and CD8) expression and cellular activation.

Acknowledgements

This work was supported by PHS grant CA57212 and grants 90B05 and 93A53 from the American Institute for Cancer Research. The authors thank John Stone for sharing unpublished data, and La-Dawn Caldwell for technical assistance.

References

[1] Salem, N. Jr., Kim, H.-Y. and Yergey, J.A. (1986) in Health Effects of Polyunsaturated Fatty Acid in Seafood (Simopoulos, A.P., Kifer, R.R. and Martin, R.E., eds.), pp. 263–317, Academic Press, Orlando.
[2] Prickett, J.D., Robinson, D.R. and Steinberg, A.D. (1981) J. Clin. Invest. 68, 556–559.
[3] Robinson, D.R., Prickett, J.D., Polisson, R., Steinberg, A.D. and Levine, L. (1985) Prostaglandins 30, 51–75.
[4] Leslie, C.A., Gonnerman, W.A., Ullman, M.D., Hayes, K.C., Franzblau, C. and Cathcart, E.S. (1985) J. Exp. Med. 162, 1336–1349.
[5] Johnston, P.V. (1985) Adv. Lipid Res. 21, 103–141.
[6] Goetzl, E.J., Wong, M.Y.S., Payan, D.G. and Chernov-Rogan, T. (1986) In Health Effects of Polyunsaturated Fatty Acid in Seafood (Simopoulos, A.P., Kifer, R.R. and Martin, R.E., eds.), pp. 239–246, Academic Press, Orlando.
[7] Fernandes, G. (1989) in Health Effects of Fish and Fish Oils (Chandra, R.K., ed.), pp. 409–433, ARTS Biomedical Publishers and Distributors, St. John's, Newfoundland.
[8] Oth, D., Mercier, G., Tremblay, P., Therien, H.M., Begin, M.E., Ells, G. and Potworowski, E. (1990) Biochim. Biophys. Acta 1027, 47–52.
[9] Stillwell, W., Wassall, S., Dumaual, A.C., Ehringer, W.D., Browning, C. and Jenski, L.J. (1993) Biochim. Biophys. Acta 1146, 136–144.
[10] Jenski, L.J., Sturdevant, L.K., Ehringer, W.D. and Stillwell, W. (1991) Lipids 26, 353–358.
[11] Fasel, N.J. and Déglon, N. (1992) Immunogenetics 35, 126–130.
[12] Irlin, Y. and Peled, A. (1992) Immunol. Lett. 33, 233–238.
[13] Williams, A.F. (1982) J. Theor. Biol. 98, 221–234.
[14] Parnes, J.R. (1989) Adv. Immunol. 44, 265–311.
[15] Veillette, A., Bookman, M.A., Horak, E.M. and Bolen, J.B. (1988) Cell 55, 301–308.
[16] Fetterhoff, T. (1990) Cytometry 11 (Suppl. 4), 87.
[17] Spector, A.A. and Hoak, J.C. (1969) Anal. Biochem. 32, 297–302.
[18] Raulet, D.H., Gottlieb, P.D. and Bevan, M.J. (1980) J. Immunol. 125, 1136–1143.
[19] Sarmiento, M., Glasebrook, A.L. and Fitch, F.F. (1980) J. Immunol. 125, 2665–2672.
[20] Jenski, L.J., Sturdevant, L.K., Ehringer, W.D. and Stillwell, W. (1993) Nutr. Cancer 19, 135–146.
[21] Pascale, A.W., Ehringer, W.D., Stillwell, W., Sturdevant, L.K. and Jenski, L.J. (1993) Nutr. Cancer 19, 147–157.
[22] Mitchell, D.C., Straume, M. and Litman, B.J. (1992) Biochemistry 31, 662–670.
[23] Hoover, R.L., Bhalla, D.K., Yanovich, S., Inbar, M. and Karnovsky, M.J. (1980) J. Cell. Physiol. 103, 399–406.
[24] Benkö, S., Hilkmann, H., Vigh, L. and Van Blitterswijk, W.J. (1987) Biochim. Biophys. Acta 896, 129–135.
[25] Muller, C.P., Stephany, D.A., Shinitzky, M. and Wunderlich, J.R. (1983) J. Immunol. 131, 1356–1362.
[26] Traill, K.N. and Wick, G. (1984) Immunol. Today 5, 70–76.
[27] Twisk, A.J.T., Rutten, F.A.J.J.L., Schornagel-Hoeben, K. and Kraal, G. (1992) Immunobiology 186, 394–409.
[28] Twisk, A.J.T., Detering, F. and Kraal, G. (1991) Immunobiology 183, 386–395.
[29] Holte, L.L., Deese, A.J.A.J., Ryba, A.J., Watts, A. and Dratz, E.A. (1990) Biophys. J. 57, 218a.
[30] Chow, S.C., Sisfontes, L., Jondal, M. and Björkhem, I. (1991) Biochim. Biophys. Acta 1092, 358–366.
[31] Robinson, D.R., Xu, L.-L., Knoell, C.T., Tateno, S. and Olesiak, W. (1993) J. Lipid Res. 34, 1423–1434.

[32] Ferguson, M.A. and Williams, A.F. (1988) Annu. Rev. Biochem. 57, 285–320.
[33] Tse, A.G.-D., Barclay, A.N., Watts, A. and Williams, A.F. (1985) Science 230, 1003–1008.
[34] Bohuslav, J., Cinek, T. and Horejší, V. (1993) Eur. J. Immunol. 23, 825–831.
[35] Dráberová, L. and Dráber, P. (1993) Proc. Natl. Acad. Sci. USA 90, 3611–3615.
[36] De Brabander, M., Nuydens, R., Ishihara, A., Holifield, B., Jacobson, K. and Geerts, H. (1991) J. Cell Biol. 112, 111–124.
[37] Edidin, M., Kuo, S.C. and Sheetz, M.P. (1991) Science 254, 1379–1382.
[38] Rothberg, K.G., Ying, Y.-S., Kamen, B.A. and Anderson, R.G.W. (1990) J. Cell Biol. 111, 2931–2938.