Dietary Lipid Modulation of Immune Responsiveness[1]

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ABSTRACT

The influence of dietary fat concentration and saturation on blastogenesis, cytotoxicity, antibody response and fatty acid composition of murine splenic lymphocytes was studied. Blastogenesis of lymphocytes from dietarily manipulated mice in response to alloantigens from control mice was significantly greater for those mice fed a diet containing minimal essential fatty acids (EFA) as the only fat source (EFA control) than those fed an EFA-deficient diet. When the dietary fat concentration was increased, blastogenic responses decreased compared to the EFA control diet. Lymphocyte-mediated cytotoxicity against allogeneic melanoma cells was greater for mice receiving diets with EFA only than for those deficient in EFA. However, cytotoxicity responses of mice fed additional polyunsaturated fat (PUF) decreased as concentration increased, whereas responses of mice fed the saturated fat (SF) diets decreased only when the dietary fat concentration was greater than 8%. As compared to diets with EFA control, direct plaque-forming cell (PFC) response was decreased for mice fed high levels of PUF and increased for mice fed high levels of SF; however, no difference in the percentage of IgM-positive cells was observed. These changes in PFC response were inversely related to the levels of linoleic acid in the lymphocyte. Thus, high levels of dietary fat, and particularly PUF, suppress lymphocyte functions when EFA requirements are met, whereas low levels (EFA control) intensify these responses. EFA deficiency, however, suppresses some lymphocyte responses. Thus, dietary lipids differentially modulate the levels of T- and B-cell responsiveness.
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INTRODUCTION

Fatty acids introduced into culture or injected subcutaneously into animals have been reported to influence immune function. Nevertheless, the exact effect of these fatty acids is a controversial issue; both enhancement and suppression of immunity have been reported. For example, PUFA dissolved in ethanol inhibited lymphocyte transformation in response to PHA (1); arachidonic acid dissolved in hexane, however, enhanced, whereas the same fatty acid dissolved in ethanol inhibited PHA-induced human lymphocyte blastogenesis (2,3). In contrast, others (4) have suggested that PUFA may not play an immunoregulatory role. Their hypothesis is based on observations that fatty acids bound to albumin did not inhibit lymphocyte transformation. However, changes of immune response after manipulation of fatty acids in vitro may not relate to changes of immune responsiveness in vivo because of the complexity of cellular and humoral interactions observed in vivo. Moreover, few studies have addressed the question of how dietary fat influences both T- and B-cell responsiveness. We (5) have previously reported that prenatal and postnatal dietary lipid manipulation can significantly influence several parameters of immune status in neonatal mice. For example, lymphocyte transformation induced by concanavalin A was significantly decreased as levels of PUF increased. In addition, the number of immunoglobulin-positive cells and serum IgG_1 and IgG_2 levels decreased with increasing fat concentration. Because of the controversial nature of fatty acid effects on immune response in vitro and the potential association between dietary fat and immune status, the purpose of the experiments reported herein were to determine the influence of dietary fat concentration and saturation on: lymphocyte blastogenesis, cytotoxicity, antibody response, and antibody response as related to the fatty acid composition of lymphocytes.

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Abbreviations: BHT, butylated hydroxytoluene; EFA, essential fatty acid; FBS, heat-inactivated, fetal bovine serum; IP, intraperitoneally; MLC, mixed lymphocyte culture; PEC, peritoneal exudate cells; PFC, plaque-forming cells; PHA, phytohemagglutinin; PUF, polyunsaturated fat; PUFA, polyunsaturated fatty acids; SF, saturated fat; and SRBC, sheep red blood cells.

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MATERIALS AND METHODS

Animals and Diets

Six week old female C57BL/6J, BALB/cAnN, and C3H/HeJ mice previously fed a stock diet (Purina Rodent Chow, St. Louis, MO) were maintained as specifically pathogen-free in an air curtain isolator and fed at a level such that each mouse had 16 kcal of metabolizable gross energy available daily. At that level, weights increased at the same rate and there was no significant difference ($p > 0.05$) between groups fed the various diets. The semipurified diets consisted of a constant amount per kilocalorie of casein, salts, vitamins and fiber. Diets then had either 0% energy from corn oil (essential fatty acid deficient) or 1.4% energy from corn oil which provided minimal EFA for each diet (6) (Table 1). To test the influence of dietary fat concentration, 4 diets contained additional 20.3% or 40.6% energy from fat; 2 of those

TABLE 1

Composition of Experimental Diets

Ingredient	1 (0 Fat)	2 (EFA)	3 (8% Fat)	4 (8% Fat)	5 (20% Fat)	6 (20% Fat)
			g/100g diet			
Casein	18.2	18.4	21.0	21.0	24.8	24.8
Salt mix[a,b]	4.5	4.7	5.4	5.4	6.4	6.4
Vitamin mix[a,c]	1.2	1.2	1.4	1.4	1.7	1.7
Fiber[d]	4.0	4.1	4.7	4.7	5.5	5.5
Cerelose	72.0	71.1	59.0	59.0	41.6	41.6
Corn oil[e,f]	0	0.5	0.6	0.6	0.7	0.7
Safflower oil[g]	0	0	7.9	0	19.3	0
Coconut oil[g]	0	0	0	7.9	0	19.3
			Percent energy			
Protein	28.0	28.0	28.0	28.0	28.0	28.0
Fat	0	1.4	20.3	20.3	40.6	40.6
Carbohydrate	72.0	70.6	51.7	51.7	31.4	31.4

[a]See Erickson, et al. (7) for composition.
[b]Provides 1.3 g/kcal of gross energy.
[c]Provides 0.3 g/kcal of gross energy.
[d]Provides 1.1 g/kcal of gross energy.
[e]Provides 1.4% of the gross energy as an essential fatty acid source.
[f]See Ossmann et al. (8) for fatty acid analysis.
[g]See Erickson et al. (5) for fatty acid analysis.

diets contained coconut oil (8.4% unsaturated bonds) and 2 contained safflower oil (89.3% unsaturated bonds). Mice were fed the experimental diets for 4 weeks before immunization, and then continued on the same diets until they were killed. All dietary groups contained at least 6 mice per group in each experiment. Separate experiments were performed 2 or 3 times.

Mixed Lymphocyte Culture (MLC)

First, to test dietary fat influences on lymphocyte transformation in response to alloantigens, spleen cells from C57BL/6 dietarily manipulated mice acted as responder cells to stimulation by BALB/c spleen cells from mice fed the stock diet. Second, to test dietary fat influences on alloantigenicity, spleen cells from dietarily manipulated C57BL/6 mice acted as stimulator cells for BALB/c responder cells from mice fed the stock diet.

Single cell suspensions of spleen cells were prepared in RPMI-1640 with 25 mM HEPES buffer, 10% heat-inactivated FBS, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at 3×10^6/ml. For the preparation of stimulator cells, spleen cells at a concentration of 10^7/ml were incubated with 50 μg/ml of mitomycin C in RPMI-1640 for 30 min at 37 C, washed and resuspended in complete medium at 3×10^6/ml. Quadruplicate cultures for each group were set up with 100 μl of stimulator and 100 μl of responder cells in a round bottom microtiter plate. Both one-way and two-way MLC were assayed. Controls included cultures of responder or stimulator cells alone as well as a coculture of syngeneic mitomycin C treated and untreated cells. All mice were treated individually. After 108 hr, 1 μCi of ^{3}H-thymidine (^{3}H-TdR; sp act 6.7 Ci/mmol) was added, and at 120 hr, the cells were collected onto glass fiber filters. The dried samples were placed into vials containing toluene and omnifluor (New England Nuclear, Boston, MA) and counted in a liquid scintillation spectrophotometer. The results are expressed as mean cpm $\pm$ SEM; the stimulation index (SI) was calculated:

$$SI = \frac{\text{CPM of allogeneic culture}}{\text{CPM of syngeneic culture}}$$

Cytotoxicity Assay

Cell-mediated cytotoxicity assays were performed in vitro as described by Benjamini et al. (9). The tumor target cell line, P51, was used for the assay. P51 was previously established from a transplantable B16 murine melanoma and propagated in culture (10-12). For the assay, P51 cells were harvested by a short trypsinization and washed; 5×10^6 cells were then injected IP into dietary-manipulated C3H mice. Ten days after injection, PEC were collected by lavage with Ca^{2+}–Mg^{2+} free Hank's balanced salt solution, centrifuged, and resuspended in RPMI-1640 with 25 mM HEPES, 10% FBS, and antibiotics. The resulting suspensions were greater than 90% lymphocytes. Tumor

target cells in their exponential growth phase were incubated at 37 C for 1 hr in RPMI-1640 containing 200 μCi of $Na_2{}^{51}CrO_4$ (sp act 128 mCi/mg). P51 cells were then washed, trypsinized, resuspended in complete medium, and 2.5×10^4 added per 38 mm^2 well (Microtest II, Falcon, Oxnard, CA). Quadruplicate cultures were set up for individual mice with an effector:target cell ratio of 50:1. Radiolabeled target cells were also plated alone. After 4 hr of coculture, aliquots of the supernatant were removed and samples counted in a gamma counter. Maximum release was determined by freeze-thawing the tumor cells 3 times. Spontaneous release was always less than 10%. The results were calculated by:

$$\% \text{ cytotoxicity} = \frac{\text{CPM released for test sample} - \text{CPM of spontaneous release}}{\text{total releasable CPM} - \text{CPM of spontaneous release}} \times 100$$

Hemolytic Plaque Assay

For measurement of primary responses, dietarily manipulated mice were immunized with 0.2 ml of 10% sheep red blood cells IP. Six days after immunization, spleen cells were assayed for antibody forming cells by the Cunningham and Szenberg modification of the Jerne plaque technique (13). Controls included samples without spleen cells and spleen cells from unimmunized mice. The former yielded no plaque-forming cells, whereas the latter yielded fewer than 5 plaque forming cells/10^6 nucleated spleen cells.

Extraction and Gas Chromatographic Analysis of Lipids

Lipid extractions were carried out by the method of Folch et al. (14), as modified by Johnson (15). For each sample, ca. 9×10^7 cells were lyophilized and 5.0 ml freshly distilled methanol containing 0.005% BHT was added. Samples were resuspended, flushed with nitrogen, and sealed with teflon tape before incubation at 55 C for 1 hr. After cooling, 10 ml freshly distilled chloroform with 0.005% BHT was added, the sample flushed with N_2, sealed, and the extraction continued at 25 C for 12 hr. Insoluble material was removed from each sample by filtration through a 0.45 μ size Millipore filter. The chloroform/methanol suspension was then washed twice with 2 M KCl, followed by one wash with 10 ml distilled water. The organic layer was passed through freshly packed anhydrous sodium sulfate columns and dried under N_2 and stored at −70 C until derivatized.

Dried lipid samples were resuspended in 3 ml petroleum ether and 300 μg of the stock C_{17} (100 μg/50 μl in isopropyl alcohol) was added to each. An aliquot was removed, placed into a round-bottomed flask, the solvent evaporated under vacuum, and 1.0 ml of 0.5 M NaOH in methanol added. The sample was heated over a steam bath for 5 min to achieve hydrolysis of the fatty acyl groups. After cooling, 1.0 ml methanolic BF_3 (14% w/v, Applied Science) was added, followed by heating at 100 C for 1 min. The solvent was evaporated under vacuum and the residue was extracted twice with 2.0 ml petroleum ether. Each extract was shaken with 1.0 ml ddH_2O, the ether layer removed carefully, combined in a round-bottomed flask, and the solvent evaporated under vacuum. The resultant methyl esters were resuspended in dichloromethane.

Gas chromatographic analyses were carried out on 1-5 μl samples of methyl esters on a Silar 10C column using hydrogen flame detection. Quantities were calculated on the basis of peaks relative to the internal standard of known fatty acid methyl esters.

Statistical Methods

The mean of replicate samples for individual mice were used for analysis. Data were initially subjected to one and two way analysis of variance (16), then to multiple-t pairwise comparisons of independent samples or (17) Scheffe's multiple range test (18). Correlation coefficients were determined for linear relationships (17).

RESULTS

Lymphocyte Blastogenesis in Response to Alloantigens

High levels of dietary fat, particularly PUF, have been shown to suppress lymphocyte responses to T-cell mitogens (5,8). We now wish to determine how dietary fats modulate lymphocyte responses to defined antigens. First, to test whether dietary fats directly influence the lymphocyte, transformation in response to alloantigens was measured. For this determination, spleen cells from dietarily manipulated mice acted as responders to stimulation by spleen cells from mice fed the stock diet (Fig. 1). Responses of $24{,}700 \pm 3{,}300$ cpm for lymphocytes from mice fed the EFA diet were significantly ($p < 0.05$) greater than responses of $9{,}200 \pm 1{,}000$ cpm for lymphocytes from mice fed a fat-free diet. With additional fat added to the diet, the levels of lymphocyte blastogenesis decreased such that at 20% concentration, responses of $14{,}500 \pm 2{,}400$ cpm for mice fed PUF diet and $14{,}200 \pm 1{,}000$ cpm for mice fed the SF diet were significantly ($p < 0.05$) less than mice fed the EFA.

Second, to test the dietary fat influences on alloantigenicity, spleen cells from dietarily manipulated C57BL/6 mice acted as stimulator cells for responding BALB/c splenic lymphocytes from mice fed the stock diet (Fig. 2). Responses of $52{,}500 \pm 1{,}740$ cpm for mice fed a fat-free diet were significantly greater than responses of lymphocytes

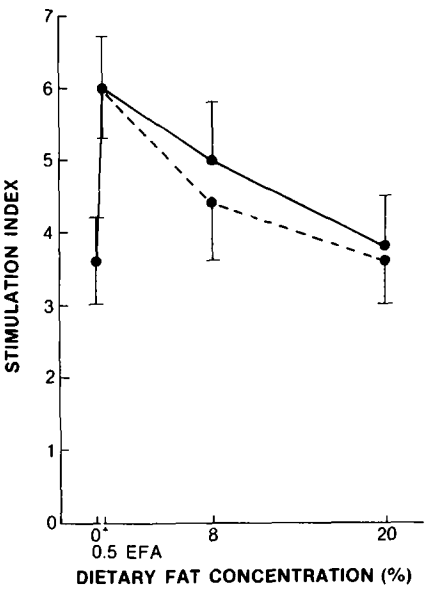

FIG. 1. Blastogenesis of lymphocytes from dietary-fat-manipulated mice in response to alloantigens from control mice. Diets contained either 0% fat (EFA-deficient) or 0.5% corn oil (minimum EFA). To test the influence of dietary fat situation, 4 diets contained additional safflower (PUF, ●— ●) or coconut (SF, ●- ●) oil. Data shown represents the mean and standard error of the mean for all mice. 36 mice were used in each individual experiment with 6 animals per diet group treated individually. All mice were assayed in quadruplicate. This was repeated 3 times.

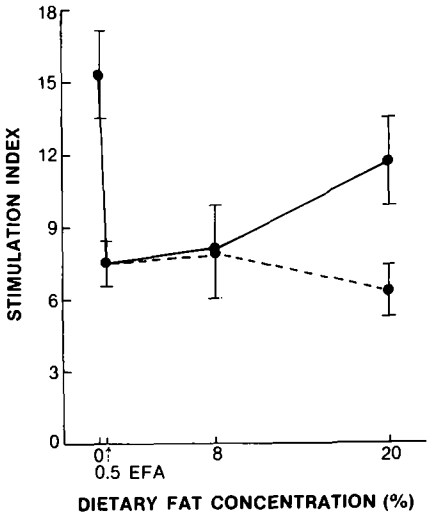

FIG. 2. Blastogenesis of lymphocytes from control mice in response to alloantigens from dietary-fat-manipulated mice. Diet contained either 0% fat or EFA. In addition to EFA four diets contained added PUF (●— ●) or SF (● ●). Data shown represents the mean and standard error of the mean for all mice. The number of animals used was the same as indicated for Figure 1.

from mice fed a diet containing fat except for those receiving a 20% PUF diet (42,000 ± 4,100 cpm). Responses of 26,500 ± 5,000; 23,600 ± 4,000; 28,000 ± 6,000; and 25,700 ± 3,000 cpm were observed for mice fed the EFA, 8% PUF, 8% SF, and 20% SF diets.

Peritoneal Exudate Cell Cytotoxicity Toward Melanoma Targets

The influence of dietary fat on PEC-mediated cytolysis was measured by radioisotope release from a standardized target cell taken from culture (Table 2). PEC from mice fed the EFA diet exhibited significantly higher levels ($p < 0.05$) of cytotoxicity than those mice receiving a fat-free diet. Cytotoxicity of PEC from mice fed additional PUF at either 8 or 20% concentration decreased compared to EFA controls. In contrast, the cytotoxic responses of mice fed the 8% SF diet were not significantly different ($p > 0.05$) than EFA diet controls but decreased in mice fed the 20% SF diet. Cytolysis mediated by PEC from mice fed the 20% PUF diet was not different as compared to cytolysis by PEC of mice fed the 20% SF diet.

TABLE 2

Cytotoxicity Mediated by Peritoneal Exudate Cells[a]

Dietary fat	% Cytotoxicity toward target cells[b]
0	20.4 ± 1.8[c]
0.5% (EFA)	27.5 ± 2.5
8% PUF	19.4 ± 2.7[c]
8% SF	25.2 ± 2.8
20% PUF	17.0 ± 1.8[c]
20% SF	20.5 ± 1.2[c]

[a]Cytotoxicity toward the P51 melanoma target mediated by allogeneic PEC from dietarily manipulated mice.
[b]Mean ± SEM for 6 mice in each dietary group.
[c]Significantly ($p < 0.05$) less than for mice fed the control (EFA) diet.

Antibody Formation by Single Cells

Dietary fat manipulation has been shown to influence serum immunoglobulin levels in neonatal mice (5). To determine whether similar lipids effect B-cell responses to specific antigens, direct plaque-forming responses to SRBC were measured (Fig. 3). The numbers of IgM plaque-forming cells/10^6 nucleated spleen cells were not significantly different ($p > 0.05$) for mice fed at either 0, 0.5, or 8% fat concentration. However, mice fed the 20% PUF diet had 276 ± 36 PFC/10^6 which was significantly

($p<0.05$) less than the number of PFC ($416\pm 40/10^6$) for mice fed the EFA diet; mice fed the 20% SF diet had 652 ± 46 PFC/10^6 which was significantly greater ($p<0.05$) than the EFA control.

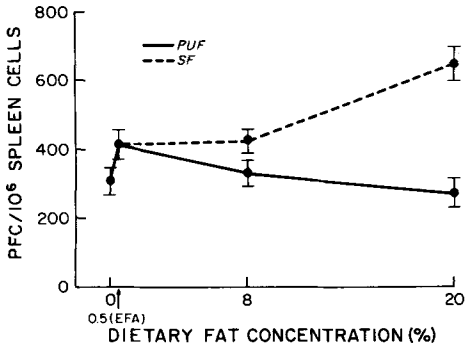

FIG. 3. Number of direct plaques from mice fed diets containing either no fat, EFA, or EFA plus additional PUF (●——●) or SF (●---●). Mice were fed various diets for four weeks before primary immunization with SRBC then maintained on the same diet until the assay on day 6. Data shown is the mean and standard error of the mean for all mice. In each experiment, 6 mice in each dietary group were treated individually; the experiment was repeated 2 times.

Fatty Acid Composition of Lymphocytes

To assess the fatty acid composition of lymphocytes after dietary manipulation and correlate potential changes with B-cell responses, fatty acid levels were determined by gas chromatography. With mice maintained on the stock diet, no significant change in the fatty acid profile of whole cell extracts was observed after immunization with SRBC as compared to control, nonimmunized mice (Fig. 4). However, by increasing either the PUF or SF concentration, the lymphocytes from mice immunized with SRBC had an increased level of palmitic and arachidonic acid as compared with the control (EFA) diet, whereas the concentration of stearic acid remained constant (Fig. 5). With increasing concentration of dietary SF, the levels of palmitoleic acid remained constant while levels of oleic and linoleic acid decreased in the lymphocyte. When the diets were supplemented with increasing amounts of PUF, the levels of linoleic acid increased with a concomitant decrease in both palmitoleic and oleic acid.

DISCUSSION

High concentrations of dietary fat and particularly PUF appear to suppress lymphocyte functions when EFA requirements are met. For example, with a T-cell function such as cytotoxicity, when mice were fed high levels of dietary fat, saturation

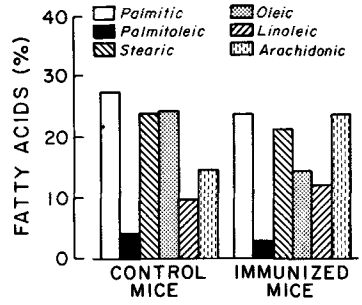

FIG. 4. Fatty acid composition of lymphocytes from immunized and control mice fed a stock diet. Values represent the means of all mice with triplicate samples for each dietary group of 6 mice. Standard errors of the mean remained <10% throughout. This experiment was repeated 2 times.

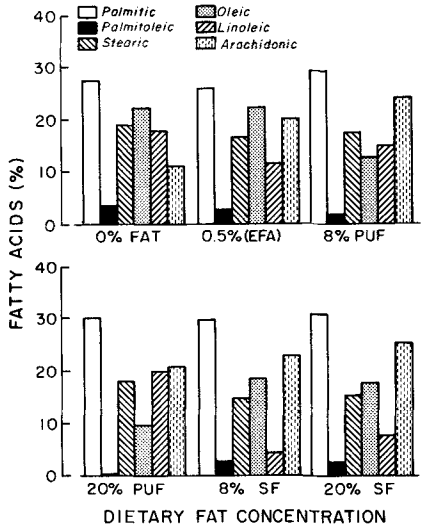

FIG. 5. Fatty acid composition of lymphocytes from dietarily manipulated mice. Animals were immunized with SRBC 6 days prior to assay. Values represent the means for all mice and at least triplicate samples for each group with 6 mice per dietary group; standard errors of the mean remained <6% throughout. This experiment was repeated 2 times.

of the fat had no influence. However, when the concentration was reduced, mice fed the PUF had suppressed responses when compared with mice fed the SF. In contrast, lymphocyte blastogenesis in response to alloantigens was not significantly influenced by saturation of the fat but by concentration only. These differences may reflect the time required for the assay. The cytotoxicity assay requires 4 hr of culture, whereas the MLC was measured after 120 hr of culture time. Thus, high levels of certain fatty acids contained in the plasma

membrane of the lymphocyte may flux into the culture medium when the cells are grown without high levels of the same exogenous fatty acid (19,20). Because it takes ca. 24 hr for cells to regain their characteristic fatty acid profiles after changing the surrounding lipid concentration, assessments of in vivo lipid modulation may be only reflected in the first 24 hr of the assay. Consequently, results of a 120 hr assay are not directly comparable with the results of a 4 hr assay and both assays may be influenced to varying degrees by the fatty acid content of the FBS. Based on the reports of other investigators (19,20), however, we would expect that the fatty acids in FBS would have little influence on a 1-4 hr assay compared to the 120 hr assay. Nevertheless, low levels of dietary fat such as EFA tend to increase the lymphocyte blastogenic responses whereas high levels tend to suppress this response. EFA deficiency, however, suppresses lymphocyte blastogenesis. Other investigators (21) have shown similar suppression of immune function; they demonstrated that subcutaneous injection of linoleic acid prolonged skin allograft survival and that a dietary deficiency of PUF resulted in immunopotentiation. In contrast, most levels of dietary fat suppressed the ability of alloantigens to stimulate blastogenesis in a mixed lymphocyte culture. Since changes in the levels of dietary fatty acids are reflected through changes in the fatty acid composition of the lymphocyte itself (22,23), the cell membrane of lymphocytes from mice fed diets with high levels of PUF or SF may have altered physical properties. A direct relationship between membrane lipid composition and surface protein mobility may exist such that the less fluid membrane lipids would result in reduced mobility of the surface proteins. This has been demonstrated in the case of patching and capping of H-2 antigens (20). Thus, changes in lateral mobility may influence the ability of H-2 antigens to stimulate lymphocyte blastogenesis in a MLC.

High levels (20%) of dietary PUF suppress direct PFC response to SRBC, whereas SF increase this response as compared to mice fed the EFA diet. This is in direct contrast to the work of other investigators (24) who have demonstrated that elevated levels of dietary PUF have no effect on the number of PFC. Since we observed no significant differences in the percentage of IgM-positive cells with dietary fat manipulation, we conclude that dietary fat influences are not due to changes in cell number but lead to a change in the frequency of B-cells responding to antigen. In addition to altering frequency, the secretory capacity of individual B-cells may be modified. Moreover, direct PFC response to SRBC appears to be inversely related to the levels of linoleic acid in the lymphocyte, i.e., as linoleic acid level increases, PFC response decreases. The correlation coefficients for all values were $r = -0.80$ ($p < 0.05$) and $r = -0.96$ ($p < 0.001$) for PUF diets only. The mechanism by which linoleic acid influences lymphocyte response is a matter of speculation. However, changes in the fatty acid composition of cell membranes can result in changes of membrane fluidity (25). These changes may adversely influence the necessary events in lymphocyte responsiveness, such as lymphocyte-antigen binding, resulting in few B-cells responding to antigen, a lower level of antibody production, or both. In view of this, we hypothesize that responses of lymphocytes to antigen may be modified depending upon the fatty acids available and that dietary fat manipulation will change the availability and thus the total fatty acid composition. Therefore, the concentration of fatty acids could affect the phase behavior of lipids, lipid-protein interaction, and conformation of glycoproteins within the membrane of the lymphocytes and immune responsiveness.

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