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EFFECTS OF ESSENTIAL FATTY ACID DEFICIENCY
AND VARIOUS LEVELS OF DIETARY POLYUNSATURATED FATTY ACIDS
ON HUMORAL IMMUNITY IN MICE

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By

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ABSTRACT

EFFECT OF ESSENTIAL FATTY ACID DEFICIENCY
AND VARIOUS LEVELS OF DIETARY POLYUNSATURATED FATTY ACIDS
ON HUMORAL IMMUNITY IN MICE

By

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Six experiments were conducted to determine the influence of an essential fatty acid deficient (EFAD) diet and dietary polyunsaturated fatty acids on humoral immunity in mice. The results indicated that:

1) Diets deficient in essential fatty acids (0% corn oil) significantly reduced humoral immunity. This reduction occurred after feeding the EFAD diet for 28 days; and preceded effects on growth or appearance.

2) Reduced immunity was demonstrated against T-cell dependent and T-cell independent antigens; and in both primary and secondary responses of mice fed the EFAD diet.

3) After 56 days of feeding the EFAD diet mice switched to the control diet (13% corn oil) for 7 days demonstrated full recovery of the humoral response.

4) Diets containing various levels of polyunsaturated fatty acids (from 2 to 70% of energy from corn oil) did not adversely affect the humoral response.

These results indicate the importance of essential fatty acids in maintaining the functional integrity of the humoral response.

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LIST OF SYMBOLS AND ABBREVIATIONS

EFAD	essential fatty acid deficient
PUFA	polyunsaturated fatty acids
CMI	cell mediated immunity
IgG	immunoglobulin G
IgM	immunoglobulin M
SRBC	sheep red blood cells
MEM	minimum essential medium

INTRODUCTION

It is well established that nutritional status influences the functional capacity of biological systems. In the case of the immune system, the complexity of this influence and its effect on health are just beginning to emerge. Increased susceptibility to certain infectious diseases has been a common observation in malnourished children (1). More detailed studies indicate that protein-calorie malnutrition as well as vitamin and mineral deficiencies, alter host resistance by decreasing immune competence (2,3). The association between nutrient intake and disease susceptibility has also been investigated with respect to dietary excesses (4,5).

In Western countries, the type and level of fat in the diet has been positively correlated with the increased incidence of certain tumors (4,6,7). In studies with rodents, elevated levels of dietary polyunsaturated fatty acids (PUFA) have been reported to enhance the development of chemically induced tumors (8-11). From this information an association between dietary PUFA and enhanced susceptibility to malignancy has been proposed (4).

Mertin has reported that PUFA inhibit lymphocyte transformation in vitro (12), and impair graft and tumor

rejection in vivo (9,13-15). Based on these observations he has proposed that PUFA suppress cell-mediated immunity (9). These views, however, have been challenged (16-19), and currently the extent to which dietary PUFA influence the immune system is open to question.

When PUFA are removed from the diet, biochemical evidence of essential fatty acid deficiency (EFAD) rapidly appears (20). This has been observed in patients receiving fat-free solutions during parenteral nutrition (21,22). At the same time, delayed wound healing and an increased incidence of infection are common observations in these patients (22).

Because of the lack of studies on the effect of EFAD on the immune response, and the ambiguity of available data on the effects of elevated levels of PUFA on the immune system, the experiments described in this thesis were undertaken. These experiments have focused on the effects of varying the level of PUFAs in the diet (from 0-70% of energy from corn oil) on the humural immune response of mice.

REVIEW OF LITERATURE

Dietary Fat and the Immune System

PUFA and Immunity. The investigation of PUFA and immunity has focused largely on the effects of PUFA, administered by subcutaneous injection, on cell-mediated immunity (CMI) (9,12-15). Using this design Mertin reported that PUFA inhibit allograft and tumor rejection capabilities in mice; and concluded that PUFA may influence disease or tumor susceptibility by inhibiting CMI (7,9,12). Several other investigators have also used subcutaneous injections of PUFA to study their effects on CMI. In general, most investigators agree that injections of PUFA are highly toxic to animals (16-18), but there is not uniform agreement with Mertin's conclusion that this treatment has immunoinhibitory effects (16-18). In other studies Mertin assessed the effects of oral administration of PUFA on CMI (9). These studies, while tending to support the hypothesis that PUFA inhibits CMI, provided no evidence on the mechanism(s) responsible for the observed results. Additional studies are clearly needed before the influence of PUFA on immunity can be unequivocally established.

The effects of PUFA on lymphocyte function in vitro have also been studied. Mertin and Hughes reported that both saturated and unsaturated fatty acids dissolved in ethanol and added to lymphocyte cultures inhibit PHA induced lymphocyte transformation (12). Other studies, however, have indicated that culture conditions (19) and the mode that fatty acids are added to the cultures (23) have a large effect on whether or not PUFA inhibit lymphocyte transformation. This suggests that a number of factors may determine this effect, and raise doubts that PUFA specifically inhibit lymphocyte function.

Malignancy. Studies associating dietary fat with enhanced tumor susceptibility first appeared in the literature almost fifty years ago (24). Since that time the influence of dietary fat on tumorgenesis has been extensively studied (25,26). A number of studies with rodents have associated dietary PUFA with enhanced development of chemically-induced tumors (27-30); but not all authors are in agreement (31). The importance of these findings to human malignancy has yet to be established (32). Epidemiological studies have been unable to associate PUFA intake with tumor incidence (33,34); and studies of PUFA and human tumor incidence have not been well controlled (35, 36). Thus, the association between dietary PUFA and cancer has been actively pursued for quite some time; but no clear-cut relationship has yet been discovered.

Multiple Sclerosis. Because there is evidence that auto-immunity may play a role in multiple sclerosis, and because low serum levels of PUFA have been found in some of these patients (37), an association between multiple sclerosis and dietary PUFA was proposed (37). While results of some human studies suggest beneficial effects of supplementing the diet of multiple sclerosis patients with PUFA (38), no conclusive evidence that dietary PUFA effects the overall course of the disease has been obtained (39). Injecting myelin basic protein (with adjuvant) into susceptible animals induces a condition called experimental allergic encephalomyelitis (40). Reports that diets high in PUFA slow the development of this disease have been interpreted to mean that dietary PUFA inhibit immune functions (33); however, direct experimental evidence for this interpretation is lacking. Whether or not this experimental model truly is representative of multiple sclerosis is open to question, and the secondary role of dietary PUFA in this condition is still unresolved.

Graft Rejection. Rejection of foreign tissues and grafts is mediated by CMI. The suggestion that PUFAs may inhibit CMI led to several studies involving kidney transplant patients. While some pilot studies reported an increase in graft survival with PUFA treatment (14), longer term studies showed no significant effect (41).

Cardiovascular Disease. Reports of increased levels of anti-milk antibodies in heart patients (42) and lipid

accumulation in auto-immune induced atheromas (43) has led to the suggestion of a link between dietary fat, immune functions, and cardiovascular disease.

Resistance to Infection. The influence of dietary fat has been studied with respect to another aspect of immunity, resistance to infection. Dogs fed high-fat diets were more susceptible to canine hepatitis virus than dogs fed a control diet (44); and chronically over-nourished dogs were more susceptible to canine virus challenge than control dogs (45). On the other hand, when dogs were fed an essential fatty acid deficient (EFAD) diet, they demonstrated increased incidences of upper respiratory, skin, and ear infections (46). Since EFAD has recently been described in patients receiving intravenous hyperalimentation (47-49), and infection is a common problem in these individuals (47), the possibility that EFAD may play a role in enhancing susceptibility to infection in these patients seems plausible.

Membrane Lipids and Lymphocyte Activation

It is important to recognize that the fatty acid composition of the diet is reflected to a significant extent in membranes of many organs (50,51). This is of particular concern in the case of the lymphocyte whose interaction with antigen and foreign cells takes place initially at the membrane surface. An alteration in fatty acid composition

of lymphocyte membranes could affect a number of events crucial to lymphocyte activation and response. The initial event in lymphocyte activation is binding of the antigen or mitogen to the lymphocyte surface (52). Following this, membrane phospholipid metabolism is stimulated (53), and the redistribution of membrane receptors into a polar cap (capping) has been observed (54). Each of these events could be affected by changes in membrane fatty acid composition.

Binding. Binding of antigen or mitogen to the lymphocyte surface is necessary to initiate activation. We are unaware of any studies describing the effect of dietary modification of membrane lipids on subsequent lymphocyte-ligand interactions. There is, however, evidence showing that hormone binding to membrane surfaces is altered when membrane lipids are disrupted with digitonin or phospholipase A treatment (55). Alterations in lymphocyte membrane fatty acids could affect binding characteristics to membrane receptors and thereby influence the ability of the lymphocyte to respond to ligands.

Phospholipids Metabolism. Lymphocyte membrane phospholipid metabolism is stimulated during lymphocyte activation (56). Marked increases in membrane phospholipid fatty acid turnover, with increased incorporation of unsaturated fatty acids into phospholipids has been reported (56-61). This is supported by studies showing increased activity of

membrane associated lysolecithin acyl-transferases (58), which catalyze the transfer of specific fatty acids into phospholipids. This enzyme has a low K_m for arachidonic acid, consistent with the large increase in arachidonate concentration in lymphocyte membrane phospholipids observed following mitogen stimulation (53,58). Evidence is accumulating to indicate that changes in membrane phospholipids following ligand binding are among the most important events in triggering lymphocyte activation (60). It would seem reasonable, therefore, to postulate that modifications in consumption of polyunsaturated fatty acids might alter cellular phospholipid metabolism and that this could be an important factor in modulating lymphocyte response.

Capping. Another early event associated with lymphocyte activation is the lateral diffusion of lymphocyte receptors through the lipid bilayer to form polar caps (54). This process, which has been extensively studied (54), is greatly influenced by membrane fluidity properties (62). The increased incorporation of unsaturated fatty acids into lymphocyte membranes following stimulation increases membrane fluidity (53) which facilitates movement of membrane receptors into caps (53). Fluidity is determined in part by the membrane PUFA/saturated fatty acid ratio (63). Since this ratio reflects the dietary intake of these fatty acids (63) it is entirely possible that dietary fat may alter lymphocyte membrane processes (such as capping) that are dependent on membrane fluidity properties.

MATERIALS AND METHODS

Animals and Diets. Male A/J mice 21 to 28 days old were housed in plastic solid-bottom cages, five mice per cage, in a temperature (24 ± 1°), light (12 hrs light per day), and humidity-controlled room. Diets and water were provided ad libitum. Formulation of the diets was on an equal energy basis with casein providing 24% of the dietary energy. Corn oil was used as a source of essential fatty acids and was assumed to contain 59.5% linoleic acid and 0.8% linolenic acid as previously determined (64). Corn oil was substituted for glucose on an equal energy basis. Composition of the diets fed is presented in Table 1.

Experiment 1. To determine the effects of various levels of dietary PUFA on the humoral antibody response four groups of mice (10 mice per group) were fed diets containing 0%, 13%, 50%, or 70% of energy from corn oil for 35 or 70 days. The antibody response was determined by the Jerne plaque assay. Body weights, spleen weights, thymus weights, and energy intake were assessed.

Experiment 2. In this experiment two aspects of the relationship between EFAD and the humoral response were examined: 1) the length of time required to observe losses in humoral immunity after switching to the EFAD diet, and

Table 1. Composition of the diets

Ingredient	Diet						
	1	2	3	4	5	6	7
Basal mix[1] (g)	35	35	35	35	35	35	35
Glucose (g)	65	63	61	57	54	20	4
Corn oil (g)	0	0.8	2.0	3.4	5	20	27
% energy from corn oil	0	2	5	9	13	50	70

[1]Basal mix contained (in g per 35 g basal): casein, 20.0; methionine, 0.3; vitamin mix, 0.4 (see reference 67); choline chloride, 0.2; mineral mix, 4.0 (see reference 68); and cellulose, 10.1.

2) the length of time required to reverse these losses by feeding the control diet. Mice were fed the EFAD diet (0% corn oil) or the control diet (13% corn oil) for up to 70 days. One group of mice was switched from the EFAD to the control diet on day 56 of the experiment. Plaque assays were performed at the times designated in Figure 1. Spleen and body weights were also recorded.

Experiment 3. To determine if reduced amounts of dietary polyunsaturated fatty acids would affect the humoral response, mice were divided into five groups (10 mice per group) and fed diets containing 0, 2, 5, 9, or 13% of energy from corn oil for 42 days. Plaque assays were performed and spleen and body weights were recorded.

Experiment 4. To examine the sequence of development of the primary antibody response in mice fed the EFAD diet (0% corn oil) or control diet (13% corn oil), plaque assays were performed 2, 3, 4, 5, and 6 days after mice were immunized (intraperitoneal injection) with $1x10^8$ sheep red blood cells (SRBCs)[5]. Spleen and body weights were also assessed.

Experiment 5. Experiments 1 to 4 focused on the effects of EFAD on the primary antibody response. To evaluate the effects of EFAD on the secondary response, mice fed the EFAD diet (0% corn oil) or the control diet (13% corn oil) were immunized with $1x10^8$SRBCs on day 42 and again on day 70. The secondary response, which is exclusively immunoglobulin G (IgG), was measured on day 75 by plaque assay;

spleen and body weights were also recorded.

Experiment 6. Optimal antibody response to SRBCs requires the interaction of T helper cells and B-cells (65). To determine the influence of EFAD on B-cell response independent of significant T-cell influence, mice fed the EFAD diet (0% corn oil) or the control diet (13% corn oil) were immunized with 10 ug of E. coli lipopolysaccharide (LPS). Plaque assays were performed by a modification of the procedure of Jacobs and Morrison (66).

Determination of Antibody Mediated Response (Jerne Plaque Assay). Antibody response was assessed by a modification of the Jerne plaque assay. Briefly, mice were injected intraperitoneally with $1x10^8$ SRBCs and killed by cervical dislocation at the times indicated in each experiment. A spleen cell suspension was prepared by gently teasing the spleen cells from the spleen capsule into sterile Hank's Balanced Salt Solution. Spleen cells were then washed, resuspended in minimal essential medium (MEM), and plated with $2x10^8$ SRBCs and MEM containing 0.6% agarose. Plates were incubated at 37^o in a humidified chamber.

Both direct immunoglobulin M (IgM) and indirect (IgG) plates were prepared in duplicate. Following an initial 90 minute incubation, the direct (IgM) plates were flooded with guinea pig complement. After reincubation for 30 minutes, plaques became visible. Each plaque represented lysis of SRBCs by antibody released by a single plasma cell in the presence of complement. The indirect plates were similarly

treated, however, to visualize IgG producing cells, rabbit anti-mouse IgG was added to the plates 30 minutes prior to the addition of guidea pig complement. Background plaques were negligible. Indirect plaques were corrected for the small numbers of IgM plaques (9%) that developed. Results were expressed as plaque-forming cells per spleen.

Determination of Lipopolysaccharide-SRBC Primary Response. A modification of the method of Jacobs and Morrison (66) was used. Mice were immunized with 10 ug of E. coli lipopolysaccharide (LPS) in 0.1 ml of a sterile solution containing 0.9% NaCl and 100 mM phosphate (pH 7.4). LPS coated SRBCs were prepared by boiling LPS for 60 minutes in 0.25 N NaOH, neutralizing the LPS containing solution with HCl, and then adding 4.0 mg of LPS to each milliliter of packed, washed SRBCs. Following a 30 minute incubation at 37^{o}, the LPS-SRBCs were washed four times with sterile phosphate buffered saline and the plaque assay performed.

Data Analysis. All data were treated statistically by either the student's "t" test, or one way analysis of variance with treatment differences determined by Tukey's test.

RESULTS

Experiment 1. To determine the effect of various levels of dietary polyunsaturated fatty acids on humoral immunity, mice were fed diets containing 0% (EFAD), 13% control), 50%, or 70% of energy from corn oil for 35 or 70 days. Body weights, energy intake, spleen weights, and thymus weights did not differ among the treatment groups after 35 days (Table 2). After 70 days, body weights, and spleen weights of mice fed the 0% corn oil diet (EFAD) were significantly lower than all other treatment groups (Table 2), but thymus weights did not differ. There was no evidence of dermal lesions at any time during the study.

Although no differences in any of the physical parameters measured were apparent after 35 days, differences in the direct (IgM) response were evident by this time (Table 3). Mice fed the EFAD diet (0% corn oil) responded only 66% as well as controls (13% corn oil): but there was no difference in the direct response between mice fed the control diet and mice fed the elevated levels of corn oil (Table 3). The indirect (IgG) response was not altered after 35 days (Table 3). Extending the feeding period to 70 days resulted in a greater loss in antibody response in the mice fed the EFAD diet. At 70 days, the direct (IgM)

Table 2. Body and organ weights of mice fed diets containing various levels of corn oil for 35 or 70 days[1]. Experiment 1.

Parameter	% Dietary Energy From Corn Oil				
	0	13	50	70	SE
35 Days					
Final Body Wt (g)	24.9[a]	24.8[a]	24.3[a]	24.2[a]	0.69
Energy Intake (Kcal/cage/day)[2]	59[a]	62[a]	62[a]	65[a]	1.22
Spleen Wt (mg)	103[a]	114[a]	101[a]	103[a]	3.98
Thymus Wt (mg)	27[a]	29[a]	29[a]	28[a]	1.45
70 Days					
Final Body Wt (g)	26.4[a]	29.3[b]	33.0[c]	31.3[bc]	0.76
Energy Intake (Kcal/cage/day)[2]	58[a]	62[a]	62[a]	65[a]	1.89
Spleen Wt (mg)	95[a]	113[b]	113[b]	126[b]	4.28
Thymus Wt (mg)	23[a]	23[a]	26[a]	26[a]	0.83

[1]Mean for 10 mice. Numbers in the same line with different superscripts are significantly different ($p < 0.05$). Mice were 28 ± 3 days of age at study onset; average initial body weight was 15.4 g.

[2]Five mice per cage.

Table 3. Primary antibody response to sheep red blood cells of mice fed diets containing various levels of corn oil for 35 or 70 days[1]. Experiment 1.

	% Dietary Energy from Corn Oil				
	0	13	50	70	SE
35 Days			plaque forming cells/spleen		
Direct (IgM)	38,430[a]	58,120[b]	47,455[b]	47,460[b]	3,319
Indirect (IgG)	52,791[a]	58,987[a]	50,716[a]	48,465[a]	1,718
70 Days					
Direct (IgM)	27,580[a]	47,200[b]	50,270[b]	48,611[b]	2,650
Indirect (IgG)	33,393[a]	45,502[b]	44,966[b]	46,477[b]	2,509

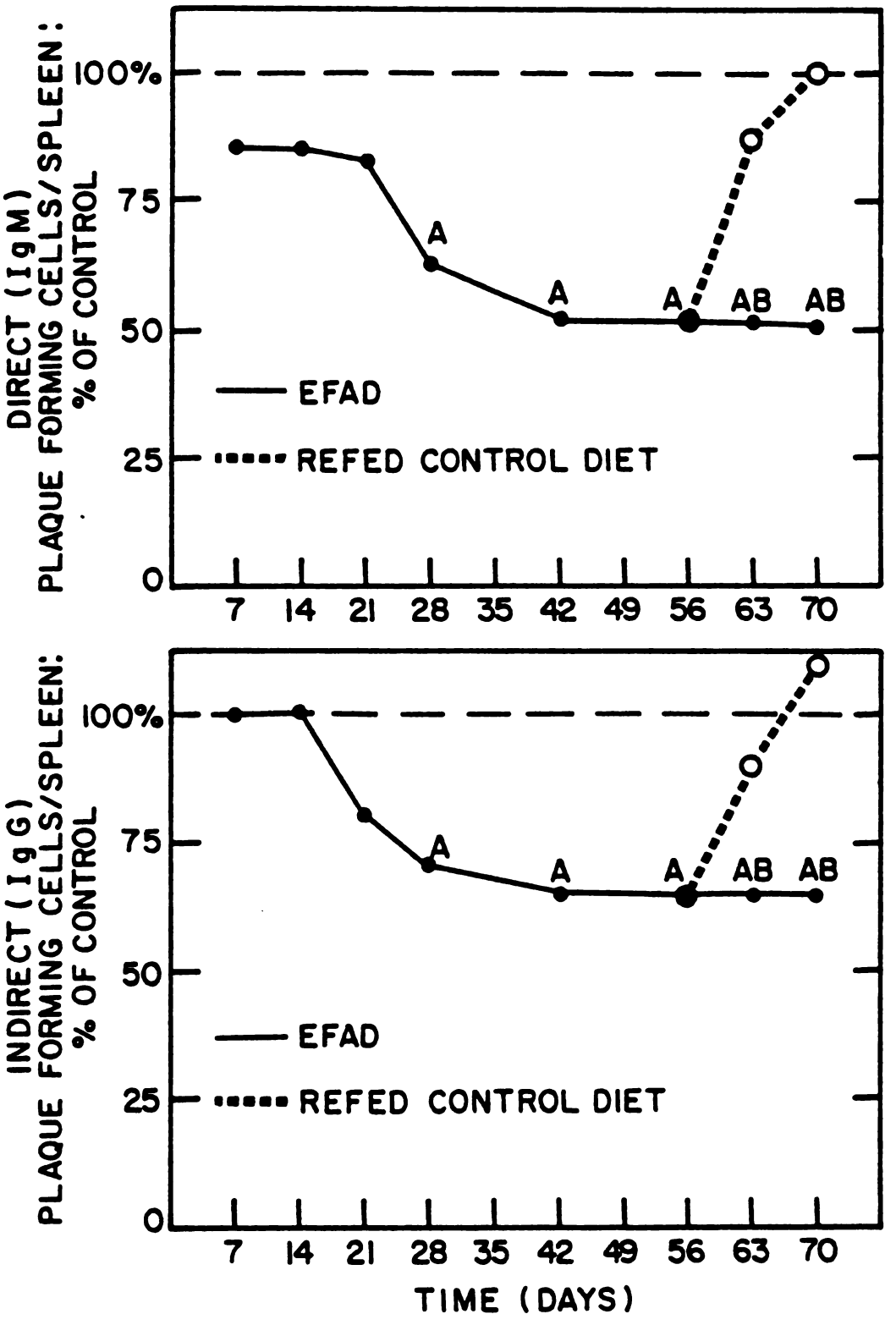
[1]Mean for 10 mice. Numbers in the same line with different superscripts are significantly different ($P < 0.05$). Mice were 28 ± 3 days of age at study onset. In the 35 day experiment, mice were immunized intraperitoneally with 1×10^8 sheep red blood cells (day 30) and response determined 5 days later (day 35). In the 70 day experiment, mice were immunized intraperitoneally with 1×10^8 sheep red blood cells (day 65) and response determined 5 days later (day 70).

response was reduced to 58% of the control response; and the indirect (IgG) response was reduced to 73% of the control values. As in the 35 day study, the antibody response of the mice fed the elevated levels of corn oil did not differ from values obtained in mice fed the control diet. This suggests that: 1) humoral immunity is significantly reduced by feeding diets deficient in essential fatty acids, but 2) humoral immunity is not adversely affected by feeding elevated levels of PUFAs.

Experiment 2. Results from Experiment 1 indicated that mice fed an EFAD diet for 35 days exhibit normal growth and appearance; but have an impaired humoral immune capacity. To determine the length of time required for an EFAD diet to significantly impair the primary antibody response, mice were fed the diet containing 0% corn oil (EFAD) for up to 70 days. The capacity of these mice to respond to SRBCs was compared to that of mice fed the control diet (13% corn oil). After 21 days both the direct (IgM) and indirect (IgG) responses of mice fed the EFAD diet were reduced to approximately 80% of the control values (Figure 1). Significant reductions were apparent at 28 days when the direct response was reduced to 64% of control and the indirect response to 71% of the controls. From 28 to 42 days both responses decreased slightly; with no further reductions apparent beyond 42 days. The significance of this plateau is not known at this time.

Figure 1. Primary antibody response of mice fed an essential fatty acid deficient diet (0% corn oil). Response expressed as percent of control (13% corn oil) response. Each point represents the mean of 10 mice. Standard errors ranged from 3 to 13% of mean values. Mean of control direct (IgM) response = 42,761 plaque forming cells per spleen; mean of control indirect (IgG) response = 44,422 plaque forming cells per spleen. A significantly different from control ($P < 0.05$). B = significantly different from refed ($P < 0.05$).

Figure 1


DIRECT (IgM)
PLAQUE FORMING CELLS/SPLEEN:
% OF CONTROL
100%
75
50
25
0
A
A
A
AB
AB
EFAD
REFED CONTROL DIET
7
14
21
28
35
42
49
56
63
70
INDIRECT (IgG)
PLAQUE FORMING CELLS/SPLEEN:
% OF CONTROL
100%
75
50
25
0
A
A
A
AB
AB
EFAD
REFED CONTROL DIET
7
14
21
28
35
42
49
56
63
70
TIME (DAYS)

To determine if the deleterious effects of the EFAD diet could be reversed, mice fed the EFAD diet were switched to the control diet after 56 days. After 7 days of feeding the control diet, both the direct (IgM) and indirect (IgG) responses were restored to control levels (Figure 1). This recovery was also apparent after 14 days, when the responses of the refed mice equalled or exceeded control levels (Figure 1). This suggests that losses in humoral immunity induced by the EFAD diet can be rapidly reversed by feeding a diet containing essential fatty acids.

Experiment 3. It was apparent from experiments 1 and 2 that a clear difference in antibody response could be demonstrated between mice fed a 0% (EFAD) or 13% (control) corn oil diet. To more closely determine the influence of the level of corn oil in the diet on humoral immunity, mice were fed diets containing 0, 2, 5, 9, or 13% of energy from corn oil for 42 days. The 2% corn oil diet was chosen to approximate the essential fatty acid requirement of the mouse for growth, (about 1.2% of energy from linoleic acid) (70). Final body weights did not differ among the experimental groups; however, the direct (IgM) response of the mice fed the diet containing 0% corn oil (EFAD) was reduced to 54% of controls (13% corn oil), and the indirect (IgG) response was reduced to 65% of controls (Table 4). Responses of mice fed the diets containing 2 to 13% corn oil did not differ. These results indicate that 1) normal

Table 4. Primary antibody response to sheep red blood cells of mice fed diets containing various levels of corn oil for 42 days[1]. Experiment 3.

	% Dietary Energy From Corn Oil					
	0	2	5	9	13	SE
	plaque forming cells/spleen					
Direct (IgM)	29,520[a]	45,560[b]	48,040[b]	47,480[b]	54,480[b]	2,550
Indirect (IgG)	35,763[a]	48,240[b]	52,366[b]	55,856[b]	54,947[b]	2,924

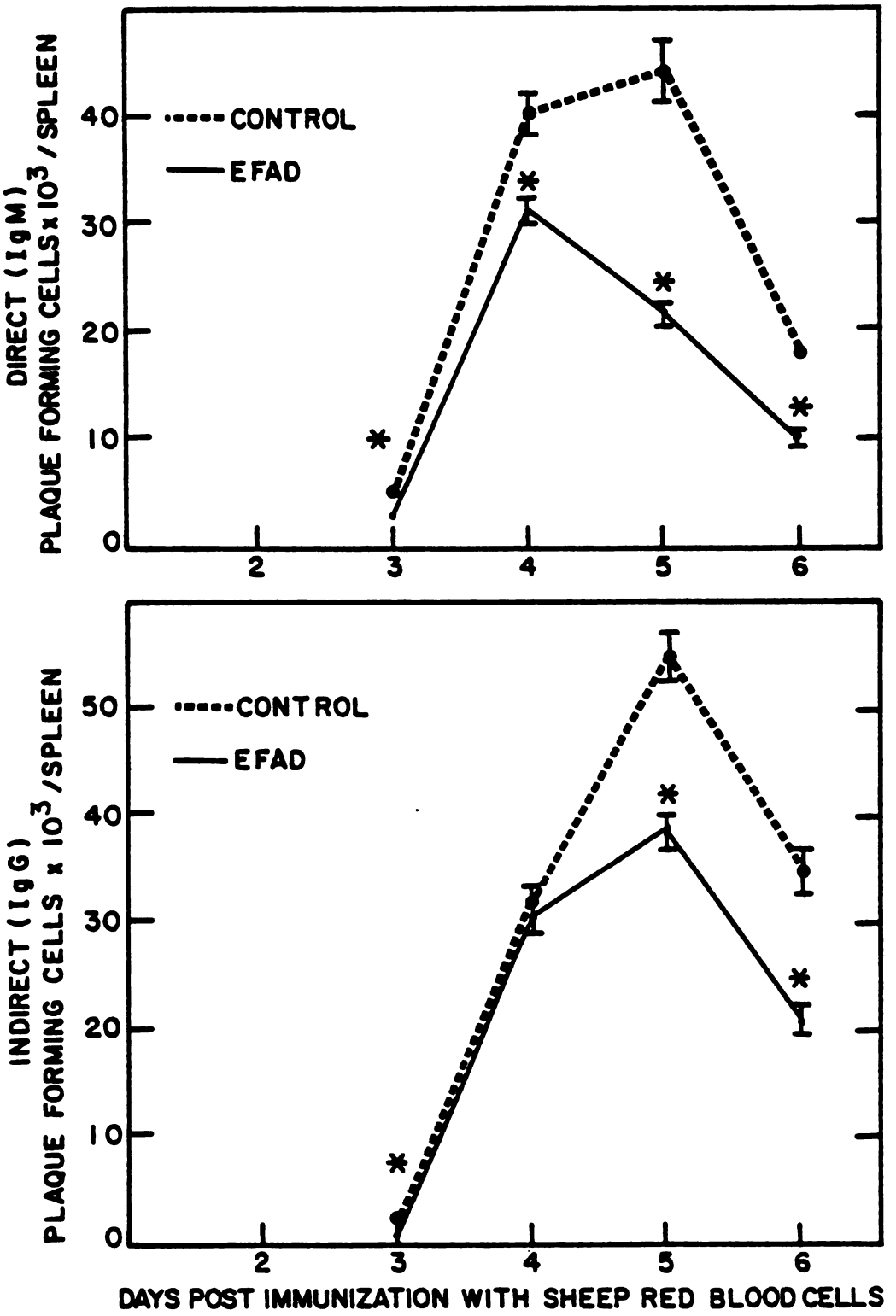
[1]Mean for 10 mice. Numbers in the same line with different superscripts are significantly different ($P < 0.05$). All mice were 21 ± 3 days of age at study onset. Final body weights did not differ among treatment groups (mean = 23.5 ± 0.5 g). Final spleen weights of mice fed 0% corn oil diet were significantly lower than the 2-13% corn oil groups (75 ± 3 vs. 99 ± 2 mg, $P < 0.05$). Mice were immunized intraperitoneally with 1×10^8 sheep red blood cells (day 37) and response determined 5 days later (day 42).

functional capacity of the humoral immune system, as well as normal growth and appearance, is maintained by feeding diets containing 2% corn oil (approximately 1.2% of energy from linoleic acid); and 2) significant losses in humoral immunity can be demonstrated in mice fed EFAD diets before a depression in growth is apparent.

Experiment 4. When A/J mice are fed a nutritionally adequate diet, optimal primary response to SRBCs occurs five days after immunization. Following this protocol, the first three experiments reported here indicated that mice fed an EFAD diet for 28 days or more produce significantly fewer plaque forming cells than control fed mice. It would, therefore, be important to assess the development of the antibody response after immunization for differences in the onset of response, sequence of appearance of IgM and IgG, and peak response. To accomplish this, mice fed an EFAD (0% corn oil) or control (13% corn oil) diet were assayed 2, 3, 4, 5, and 6 days following immunization with SRBCs. The direct (IgM) response of the mice fed the EFAD diet was significantly reduced from the earliest detectable day (day 3) through to day 6 (Figure 2). Compared to values for mice fed the control diet, mice fed the EFAD diet produced 52% of control values on day 3, 79% on day 4, 54% on day 5, and 61% on day 6. Also, peak (IgM) response of the EFAD mice occurred on day 4, compared to day 5 for controls. The significance of the altered peak response is not known at

Figure 2. Primary antibody response 2, 3, 4, 5, and 6 days after intraperitoneal immunization with $1x10^8$ sheep red blood cells. Mice were fed an essential fatty acid deficient (0% corn oil) or control (13% corn oil) diet for 56 days. Each * indicates significant differences ($P < 0.05$) between the two treatment groups. Each point represents the mean (± SEM) response of 8 mice.

Figure 2


DIRECT (IgM)
PLAQUE FORMING CELLS x 10^3 / SPLEEN
CONTROL
EFAD
0
10
20
30
40
2
3
4
5
6
INDIRECT (IgG)
PLAQUE FORMING CELLS x 10^3 / SPLEEN
CONTROL
EFAD
0
10
20
30
40
50
2
3
4
5
6
DAYS POST IMMUNIZATION WITH SHEEP RED BLOOD CELLS

this time.

Low levels of IgG production were also detectable by day 3. At this point the indirect (IgG) response of the EFAD mice was only 54% of control values. By day 4, however, the indirect response of the two groups did not differ. Peak indirect (IgG) responses of both EFAD and controls were observed on day 5, with the EFAD peak response at 64% of controls. At day 6, EFAD response was 68% of controls.

It has been known for some time that the switch from IgM to IgG production requires T-cell helper function (65). Results from this experiment suggest that consumption of the EFAD diet, which consistently reduced IgM production, may be less effective in reducing IgG production, and raises the possibility that EFAD may impair B-cell function more than T-cell function.

Experiment 5. An essential component of humoral immunity is the secondary or "memory" response. This response occurs after a second encounter with the same antigen and is characterized by a high rate of antibody production which is exclusively IgG. To determine the effect of EFAD on the memory response, mice fed diets containing 0 or 13% corn oil received immunizations with SRBCs on days 42 and 70. The response of mice fed the EFAD diet was only 57% of the controls (Table 5). This indicates that EFAD further compromises humoral immunity by reducing the generation of memory cells, and therefore reducing the memory response.

Table 5. Secondary antibody response to sheep red blood cells of mice fed an essential fatty acid deficient (0% corn oil) or control (13% corn oil) diet for 75 days[1]. Experiment 5.

	% Dietary Energy From Corn Oil		
	0	13	SE
	plaque forming cells/spleen		
Indirect (IgG)	98,000[a]	171,900[b]	12,168

[1]Mean for 10 mice. Numbers in the same line with different superscripts are significantly different ($P < 0.05$). Mice were 21 ± 3 days of age at study onset. Response assessed (day 75) following primary immunization (day 42) and secondary immunization (day 70) with 1×10^8 sheep red blood cells. Final body weights (23 ± 0.7 vs. 27 ± 0.5 g) and spleen weights (116 ± 8 vs. 138 ± 5 mg) were significantly lower ($P < 0.05$) in the essential fatty acid deficient group compared to controls.

Experiment 6. Optimal response to SRBCs requires interaction between both major populations of lymphocytes; T-cells (thymus derived) and B-cells (bone marrow derived). Certain antigens (T-cell independent antigens) have been identified that do not require significant T-cell helper influence to elicit antibody response. In these cases, the response is largely B-cell dependent, and the class of antibody produced is exclusively IgM (66). In experiments 1 to 4 impairment of the direct (IgM) response of EFAD mice appeared to be greater than the indirect (IgG) impairment. This suggests that the EFAD may result in a greater loss in numbers or in functional capacity in B-cells than in T-cells. To assess B-cell function without significant T-cell helper influence, mice fed EFAD and control diets were immunized with E. coli lipopolysaccharide (LPS), a primarily T-cell independent antigen. In a modification of the plaque assay, LPS was absorbed to the SRBC surface and the plaque assay performed as usual. Under these conditions, the primary response of the mice fed the EFAD diet was reduced to 62% of controls after 42 days, and 58% of controls after 56 days (Table 6).

These results are consistent with our previous assessments of the direct (IgM) response and support the hypothesis that EFAD significantly impairs B-cell function. The extent to which EFAD effects T-cell function is not known at this time.

Table 6. Primary antibody response to lipopolysaccharide-sheep red blood cells of mice fed an essential fatty acid deficient (0% corn oil) or control (13% corn oil) for 42 or 56 days[1]. Experiment 6.

	% Dietary Energy From Corn Oil		
	0	13	SE
	plaque forming cells/spleen		
42 Days			
Direct (IgM)	38,081[a]	61,450[b]	4,642
56 Days			
Direct (IgM)	29,000[a]	49,675[b]	3,768

[1]Mean for 10 mice. Numbers in the same line with different superscripts are significantly different ($P < 0.05$). Mice were 21 ± 3 days old at study onset. Response assessed four days after intraperitoneal immunization with 10 ug of lipopolysaccharide. In the 42 day experiment final body weights (22.1 ± 0.5 g) did not differ, however, spleen weights of the essential fatty acid deficient mice were significantly lower ($P < 0.05$) than controls (93 ± 2 vs 111 ± 6 mg). In the 56 day experiment, final body weights were significantly lower in the essential fatty acid deficient group (22 ± 0.6 vs 26 ± 0.6 g) ($P < 0.05$) and final spleen weights were also significantly lower (89 ± 7 vs. 113 ± 3 mg) ($P < 0.05$).

DISCUSSION AND CONCLUSIONS

In recent years numerous investigations have been directed towards understanding the relationship between diet and immunity. Results from these studies have indicated that protein-calorie malnutrition (1-3), as well as various vitamin and mineral deficiencies (71-73) reduce immune-competence. The results presented here indicate that EFAD also causes a significant reduction in immunity. Significant losses in humoral immunity were observed in EFAD mice challenged with T-cell dependent and T-cell independent antigens. These losses occurred before any changes in growth or appearance, and indicate that decreased immune-competence precedes the gross symptoms of EFAD.

Because PUFAs have been reported to inhibit various aspects of immunity, we also investigated the effects of various levels of dietary PUFAs on the humoral response. In these experiments, corn oil was used as the dietary source of PUFAs, and the amount of corn oil varied from 2 to 70% of dietary energy. The 2% corn oil diet was chosen to approximate the EFA requirement of the mouse for growth (approximately 1.2% of energy from linoleic acid) (70). Under the conditions of these experiments, diets containing as much as 50 to 70% of energy from corn oil had no effect

on the antibody mediated response. It is concluded from these experiments that EFAD significantly reduces humoral immunity in the mouse; while elevated levels of dietary PUFAs have no effect. Several explanations can be offered for these findings.

In mice fed the EFAD diet, losses in humoral immunity were generally accompanied by a decrease in spleen weight, and a proportional decrease in splenic lymphocyte numbers (averaging approximately 10^6 lymphocytes per mg spleen weight in both EFAD and control mice). When results were expressed on a per million spleen lymphocyte basis, rather than on a total organ basis, the EFAD response remained significantly lower than controls. Thus, in addition to the reduced numbers of lymphocytes populating the spleen, there was a significant reduction in the ability of the residual lymphocytes to respond to antigen. Interaction between antigen and specific lymphocyte membrane receptors initiates lymphocyte stimulation and response. Since essential fatty acids are important components of membranes, the effects of EFAD on immunity could result from alterations in membrane structure induced by EFAD.

It has been established that the lipid composition of the diet is reflected in tissues and membranes (50, 74); and that the physical properties of the membrane lipids affect cell function (50, 63). Feeding an EFAD diet has been shown to decrease lymphocyte linoleic and arachidonic acid content (37), and to decrease membrane PUFA

concentration (50). This decrease in membrane PUFAs could result in a decrease in membrane fluidity (63), which has recently been shown to be important in "capping" (62), and other membrane events associated with lymphocyte stimulation (75). Thus, changes in lymphocyte membrane fatty acid composition, resulting in a decrease in membrane fluidity, could impair lymphocyte activation and result in fewer numbers of cells capable of responding to antigen. In addition, recent work by Ferber and Resch (53) showed that marked changes in lymphocyte membrane fatty acid turnover, with increased incorporation of unsaturated fatty acids (specifically linoleic and arachidonic acid) occur upon activation of the lymphocyte (53). It would seem reasonable, therefore, that the reduced availability of linoleic and arachidonic acids, as occurs with EFAD, could reduce lymphocyte activation and response.

We are not aware of any previous studies on the effects of dietary PUFAs, or EFAD, on humoral immunity. However, the relationship between PUFAs and cell-mediated immunity has been studied (9, 33). Results presented here indicate that elevated levels of dietary PUFAs do not adversely affect humoral immunity; and that EFAD significantly reduces humoral immunity. This is not in agreement with the results of Mertin, who reported that PUFAs inhibit cell-mediated immunity, and that "PUFA deficiency" potentiates cell-mediated immunity (9). It is important to recognize

that the experiments reported here assessed humoral immunity, as opposed to Mertin, who assessed cell-mediated immunity (9). In addition, our studies focused on the effects of PUFAs as supplied in the diet; whereas in the majority of Mertin's studies, PUFAs were administered by subcutaneous injection or stomach tube (9, 13-15). The extent to which these differing routes of administration may stress the animals, and in this way affect results, is not known.

In summary, little is currently known about the relationship between nutritional status and immune functions. This is particularly true for essential fatty acids. Results presented here indicate that EFAD profoundly reduces humoral immunity. This supports the hypothesis that essential fatty acids play a crucial role in maintaining the functional integrity of the humoral immune system. Although the mechanisms for this effect are not yet known, essential fatty acids are important components of membranes and lipoproteins, and are precursors of prostaglandins. Because each of these could affect lymphocyte activation (62, 75, 76), essential fatty acids are in a unique position to influence immunity.

RECOMMENDATIONS

The overall purpose of these experiments was to assess the influence of an essential fatty acid deficient (EFAD) diet, and various levels of dietary polyunsaturated fatty acids on humoral immunity. Based on the results, it is apparent that EFAD can significantly reduce the humoral response, but diets containing elevated levels of polyunsaturated fatty acids have no effect. This indicates that EFAD impairs B-cell function, but does not directly address the effects of EFAD, or dietary PUFAs, on T-cell function. Thus, future experiments should be designed to assess the effects of EFAD, and various levels of dietary PUFAs, on in vivo cell-mediated immunity (CMI). In addition, in vitro experiments should be carried out to more closely determine the role of essential fatty acids in lymphocyte function. To accomplish this, the role of essential fatty acids in the sequence of events leading to lymphocyte activation should be studied. These events include: ligand binding to the lymphocyte surface, membrane phospholipid fatty acid metabolism, receptor "patching" and "capping", and RNA and DNA synthesis during blastogenesis. To more closely monitor the role of essential fatty acids in lymphocyte activation, studies should be carried out using a variety of culture

conditions including standard medium supplementation with fetal bovine serum (FBS), supplementation with sera from mice fed the various experimental diets, serum-free cultures, and serum-free cultures with selected fatty acids.

Information obtained from the studies described in this thesis, as well as those proposed for future consideration, should help us understand how essential fatty acids are involved in modulation of the immune system and secondly, should provide a model system whereby influences of other fatty acids on the immune system can also be studied.

A better understanding of the role of dietary fat in human health will make a significant contribution to future decisions involving recommendations for the type and amount of fat in the American diet (77). Also, because these studies will focus on the effects of dietary fat at the cellular level, additional insights will be gained regarding the role of diet in membrane-associated events leading to biological activity in the cell.

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