EFFECTS OF ESSENTIAL FATTY ACIDS ON IMMUNE FUNCTIONS IN MICE

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ABSTRACT

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Recent reports indicate that polyunsaturated fatty acids (PUFA) impair immunity. This research was aimed at investigating the effects of essential fatty acid deficiency (EFAD) and dietary PUFA on immune functions. Delayed type hypersensitivity was reduced 30% in mice fed the EFAD diet compared with mice fed the control or high PUFA diet. Numbers of splenic and thymic mononuclear cells and proportions of T and B cells were unaffected by diet.

In long term cultures (60-108 hours) containing fetal calf serum (FCS), ^{3}H-thymidine incorporation into lymphocytes from mice fed the three diets was similar in response to mitogens and to one-way mixed lymphocyte reactions (MLR). In short term cultures (1-4 hours) containing FCS, Con A induced ^{14}C-choline and ^{3}H-inositol incorporation into lipids was also unaffected by diet. But when a lipid free media was used, Con A induced ^{14}C-choline and ^{3}H-inositol

incorporation into lipids of lymphocytes from mice fed the EFAD diet was less than 50% of the incorporation into lymphocyte lipids of the other treatments. After 4-8 hours in culture, treatment differences were no longer detectable.

In lipid-free cultures accumulation of aminoisobutyric acid (AIB) by lymphocytes from mice fed the EFAD diet was reduced by 30% at all time points (30-240 minutes) compared with the other treatments. AIB uptake Vmax was similar across the treatments, but the Km for the Na^+ dependent component of AIB uptake was 70-85% higher in Con A activated EFAD lymphocytes compared with the other two treatments.

Viability, ^{3}H-uridine incorporation into RNA, and ^{14}C-leucine incorporation into protein were unaffected by diet, but the protein content of lymphocytes from mice fed the EFAD diet was nearly double that of the other two groups.

These results indicate that: (1) EFAD impairs in vivo immune functions, but high PUFA diets do not, (2) EFAD does not alter lymphocyte populations, or proliferative responses to mitogens and MLR, but, (3) phospholipid metabolism and neutral amino acid transport are impaired in EFAD lymphocytes. These impairments may play a role in the EFAD effects on in vivo immunity.

This Dissertation is dedicated to my Dad

who taught me a whole lot of

things you can't learn from books

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INTRODUCTION

Essential fatty acids (EFA) are structural components of biological membranes and precursors in the synthesis of prostaglandins and related lipids (1). In recent years EFA metabolism in immunocompetent cells has been intensely studied (2-5), leading to major advances in tracing and identifying EFA metabolites and their biological effects. In spite of this progress in EFA metabolism, basic information about the relationship between dietary EFA and immune functions is generally lacking. Several experimental and clinical observations indicate the importance of obtaining a better understanding of the relationship between dietary EFA and the immune response. Some of these studies have been reviewed previously (6), and others will be discussed in the literature review section.

A few authors have attempted to look directly at the relationship between dietary EFA intake and immune functions. But one of these authors used diets that differed in energy content (7), one did not use quantitative assessments of immune responses (8), and others administered fatty acids by injection, rather than in the diet (9-11). In spite of these difficulties these authors generally concluded that "PUFA excess" impaired immunity and "PUFA

deficiency" enhanced immunity (10).

My previous studies were not in agreement with these reports (12). I found that an essential fatty acid deficient (EFAD) diet reduced in vivo plaque forming responses, while elevated levels of dietary PUFA had no adverse effects on these responses (12).

The overall purpose of the experiments reported here was to further clarify the relationship between dietary EFA and the immune response and to investigate the cellular defect underlying the EFAD induced impairment of in vivo immune functions.

OVERVIEW AND OBJECTIVES

Previous experiments showed that the humoral immune response of mice was sensitive to dietary EFAD (12). But these findings differed from those reported by others. The differences between experimental results, however, may be a reflection of differences in experimental protocol. For example, as opposed to subcutaneous injection (9-11), or gavage (10), my approach was to administer the EFA as a part of an isocaloric diet. Thus, this would seem to be the more physiological approach.

I further studied the effects of the experimental diets on another in vivo immune function, delayed type hypersensitivity. These results were in close agreement with those from the plaque assay experiments (13). Since results from both in vivo studies were roughly equal, the remaining experiments were aimed at investigating the mechanism by which EFAD alters immune functions.

The majority of the in vitro experiments performed used mitogens to assess lymphocyte activation. The most obvious reason for this is that large numbers of lymphocytes within a population respond to a particular mitogen, as opposed to the small number that respond to a specific mitogen. With a large number of responding cells, more

and varied types of experiments can be performed. Although some differences exist between mitogen activation and antigen activation, there are many similarities: (1) both activate lymphocytes by binding to the lymphocyte surface, (2) neither require internalization for cell activation, (3) both initiate a series of cellular events that can be monitored during activation, and (4) after stimulation, both lead to some functional response by the activated cell (i.e., antibody production, lymphokine production) that can be measured under certain experimental conditions (14).

Significant differences do exist, however. Mitogens activate large numbers of T or B cells, whereas, antigens activate only a few cells. Also, the initiating event in mitogen activation differs from antigen activation. Mitogens activate lymphocytes in a nonspecific manner, triggering the cell by cross-linking membrane glycoproteins with certain sugar residues (14). Antigens, on the other hand, trigger lymphocytes by binding to specific receptors on the lymphocyte surface (15-16). On B cells, the antigen receptor is surface Ig (15); on T cells, the antigen receptor is an Ig-like protein complex (16). In addition, the antigen activated lymphocyte is subject to a number of regulating influences in vivo that are not duplicated by the mitogen activation in vitro. Furthermore, since certain lymphocyte cell functions do not require blast cell formation (3), defects in lymphocyte function may not be detected in

standard blast cell formation assays.

Despite these drawbacks mitogens continue to be used in a variety of studies investigating cell activation (17). Mitogens are particularly useful in studies where basic information about the relationship between a treatment and lymphocyte activation is lacking. On this premise it would seem that mitogens are valid tools for use in the present study.

Objectives

Several recent reports have raised the possibility that dietary fat, particularly PUFA, may contribute to the etiology of some infectious and chronic diseases by influencing the immune system. The overall goal of this research, therefore, was to investigate the relationship between dietary PUFA and the immune response. To accomplish this, several specific objectives were outlined. The first objective was to determine the effect of various levels of dietary PUFA, including EFAD, on in vivo immune functions. The second objective was to investigate the nature of this effect using cell culture.

As the project developed it became clear that EFAD impaired in vivo immune functions, but elevated levels of dietary PUFA had no effect. As a result, much of the cell culture work was aimed at investigating the cellular defect induced by EFAD in an effort to determine the role of EFA in lymphocyte responses.

LITERATURE REVIEW

Essential Fatty Acids: Historical Perspective

Although EFA were first described over 50 years ago by George and Mildred Burr, the precise metabolic functions of EFA are still poorly understood (1,18). One reason for this is that it took some time before EFAD was recognized as a condition relevant for study in humans. An early attempt by W. R. Brown, an associate of the Burrs to induce EFAD in himself proved unsuccessful, although he reportedly remained on a fat free diet for six months (18). Sometime later, Hansen, et al. (19) were successful in inducing severe clinical symptoms of EFAD in otherwise healthy infants. But because of the prolonged feeding period required to induce the symptoms, this only seemed to further characterize EFAD as an improbable nutrient deficiency. Following the development of total parenteral nutrition (TPN) by Dudrick, Vars, and colleagues (20), however, the first reports of EFAD began to appear in the literature. First, reports of EFAD in infants receiving TPN appeared (21), followed by reports of EFAD in adult patients after prolonged TPN treatment (22).

Further study of the TPN patients revealed that the EFAD accompanying TPN involved a complex interaction between

the EFAD solution and metabolism. It was found that changes in metabolism associated with the continuous infusion of the hypertonic glucose solutions played an important role in the early onset of the EFAD symptoms (23). The high rates of glucose infused resulted in a continuous and potent stimulii for insulin secretion. With plasma insulin levels chronically elevated, adipose tissue lipolysis was severely reduced. This effectively isolated TPN patients from their own sources of EFA and many patients exhibiting clinical and biochemical evidence of EFAD had normal stores of EFA in adipose tissue (23). This was confirmed by several studies and one solution to the problem has been the use of "cyclic hyperalimentation", in which hyperalimenated patients undergo periods of reduced, or limited infusion allowing for some lipolysis and release of EFA (23). Another strategy has been the use of solutions containing EFA (24). These solutions, because they are isotonic, can be delivered through a peripheral vein, thus avoiding placement of a central catheter. At present, EFA-containing solutions such as Intralipid are currently being used in clinical situations requiring prolonged TPN.

One problem associated with studies of EFAD has been defining its symptoms. Clinically, external symptoms such as dermatitis, weight loss, reduced growth, and increased water loss leading to increased water consumption are commonly described consequences of EFAD (1). Other abnormalities that have been described include erythrocyte

fragility (1), abnormal mitochondrial function (25), alterations in membrane enzymes (26-28), and increased synthesis of surface glycoproteins (29). For the most part these defects appear to be associated with the structural function of EFA in biological membranes, but this is not firmly established.

Another observation common to EFAD animals is fatty liver (1). It has been proposed that fatty liver in EFAD may be due to abnormalities in the formation of very low density (VLDL) and/or in the secretion of VLDL (30,31). However, a recent study by Huang and Williams (32) found that hepatic triglyceride secretion was actually higher in EFAD rats than in control rats. Thus, the origin of fatty liver in EFAD remains controversial. But these studies do point out that EFA function in normal lipid transport and metabolism.

Another aspect of EFA metabolism receiving increased attention in recent years is the oxidative transformation of arachidonic acid (AA) to prostaglandins and related lipids (3,4,33). The biochemistry and physiological significance of these compounds in regulating immune functions are discussed later in the literature review.

Major advances in EFA biochemistry have occurred largely because of the development of sophisticated chromatographic methods, particularly gas liquid chromatography (GLC). The development of GLC in the early 1950s enabled

investigators to obtain more detailed structural information about a wide variety of compounds, particularly fatty acids.

While understanding the chemistry of EFA is important, much remains to be learned about the physiological roles of EFA. In the remainder of this literature review, further aspects of EFA metabolism and its physiological and immunological effects will be discussed.

Essential Fatty Acid Metabolism

Over 50 years ago Burr and Burr described the essential fatty acids as a group of three polyunsaturated fatty acids: linoleic acid,18:2ω6; linolenic acid 18:3ω3, and arachidonic acid,20:4ω6 (1,18). Since that time, however, it has become apparent that linolenic acid is not a dietary essential. In a recent investigation of linolenic acid requirements, rats were fed a linolenic acid free diet through 2-3 generations (34). Normal growth, organ weights, and reproduction, but lower phospholipid lineolenic acid content were found in the linolenate free group (34). It is known, however, that linolenic acid incorporates in membrane phospholipids of the central nervous tissue and retinal photoreceptors (35), but as yet a unique function for linolenic acid, or cellular defect induced by its absence, is lacking.

Unlike linolenic acid (n=3) numerous studies over the years have demonstrated the essentiality of the n=6, or

ω6 PUFA (1,18). Mammals cannot synthesize 18:2ω6, however, if 18:2ω6 is provided in the diet, most mammals can synthesize 20:4ω6 from this precursor by a series of elongation and desaturation reactions (Figure 1). Animals that are exceptions to this are cats, lions, and turbot (18). They must obtain their EFA as 20:4ω6 because they lack the necessary delta^{6}-desaturase enzyme (18). In most mammals, therefore, tissue 20:4ω6 content results from dietary intake of 20:4ω6, or conversion from 18:2ω6 (1,18).

Intracellular pools of free 20:4ω6 (AA), however, are very low. Therefore, cleavage of AA from membrane phospholipids is the initiating step in the synthesis of PG and related lipids (2,6,36). This membrane cleavage appears to be the rate-limiting step in further AA metabolism and it is catalyzed by phospholipase A_2 in most tissues (2,6,36). In platelets, however, this reaction is catalyzed by either phospholipase A_2 (37), or phospholipase C and diglyceride lipase (38). The newly released AA is then rapidly metabolized by the first reaction in prostaglandin synthesis, PG endoperoxide synthetase (33,36). This enzyme complex is composed of a cyclooxygenase which catalyzes the cyclization of AA to form a C_{15} hydroperoxy, C_9, C_{11}, endoperoxide called PGG_2. The second component of the PG endoperoxide synthetase complex is a peroxidase which catalyzes the reduction of the C_{15} hydroperoxy group to a hydroxyl, forming PGH_2 (33,36). At this point PG synthesis

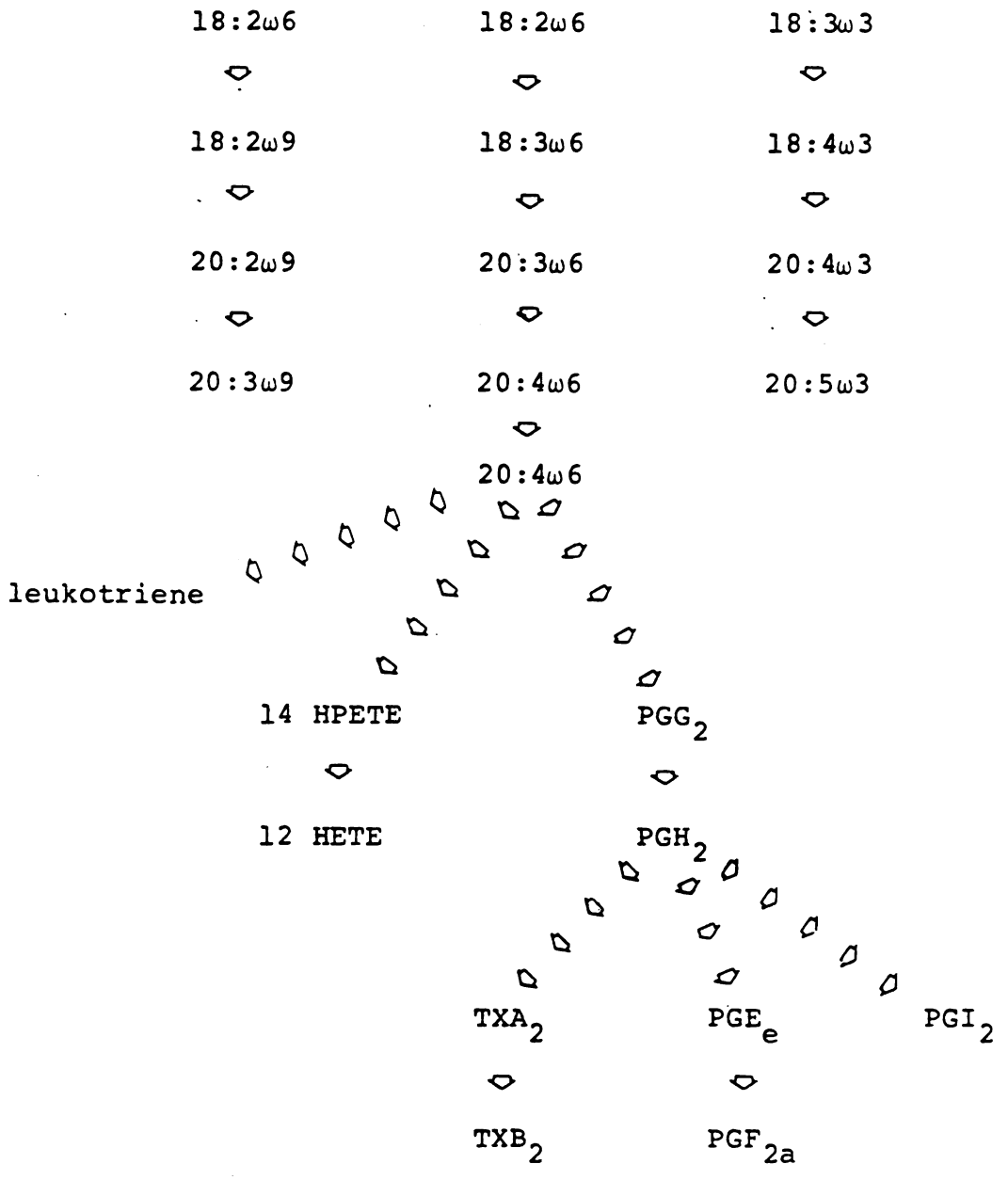

Figure 1. Unsaturated fatty acid metabolism.

may proceed to form PGE_2, PGF_{2a}, or in the case of vascular endothelial cells PGI_2, or prostacyclin (Figure 1).

Platelets metabolize PGH_2 through the enzyme thromboxane synthetase to form a highly unstable proplatelet aggregating factor called thromboxane A_2 (TXA_2). TXA_2 is rapidly degraded to its inactive form TXB_{2x} (33,36).

Recent studies have shown that ingestion of a diet high in 20:5ω3, such as the high fish diet of Greenland eskimos, has profound effects on tissue PUFA levels and EFA metabolism (35). Serum lipids from these eskimos contain elevated levels of phospholipid 20:5ω3, and relatively low levels of 20:4ω6. They have a low incidence of cardiovascular heart disease, and markedly elevated clotting times (35). This is believed to result from the substitution of 20:5ω3 for 20:4ω6 in platelet phospholipids. As a result of this substitution, TXA_3 is produced in stimulated platelets instead of TXA_2 (Figure 1). In contrast to the potent proaggregating effects of TXA_2, TXA_3 impairs platelet aggregation. Overall, incorporation of 20:5ω3 into platelet phospholipids reduces platelet aggregation by at least 3 ways: (1) 20:5ω3 competes with remaining 20:4ω6 for cyclooxygenase, thus, increased 20:5ω3 decreases 20:4ω6 metabolism to TXA_2, (2) increased 20:5ω3 increases the production of antiplatelet aggregating TXA_3, and (3) 20:5ω3 is metabolized in endothelial cells to PGI_3, which appears to be a potent antiaggregating PG, possibly even more potent

than the normally produced PGI_2.

Recent progress in the biochemistry of EFA metabolism has led to the elucidation of an entire new pathway of EFA metabolism. This pathway, which is initiated by the enzyme lipoxygenase, metabolizes AA to monohydroperoxy and monohydroxy eicosatetraenoic acids (HPETE and HETE). Some of these recently described compounds are potent chemotactic agents (2-4), and they appear to be generated early in the activation of immunocompetent cells (2-4), possibly functioning as intracellular messengers (2-4). One of these, 12-HPETE, enhances PMN hexose transport (39,40).

Other compounds formed through the lipoxygenase pathway are the dihydroxy metabolites of AA (2,3,41,42). Since most of the evidence for the existence of these dihydroxy AA compounds comes from studies with leucocytes they have been named Leukotrienes (3). They appear to influence a variety of functions in immunocompetent cells and one, Leukotriene C, appears to have the biological function of slow-reacting substance of anaphalaxis (SRSA) (2,4,41,42), confirming an earlier report that SRSA was derived from AA (43).

EFA, Prostaglandins and Cells of the Immune System

A large body of evidence indicates that macrophages of several species actively metabolize AA to a variety of

metabolites, including most PG and TX (2-4). The data for lymphocytes, however, is less clear (3). One problem in all studies of purified cell populations is obtaining that population without contamination by other cell types (2). Despite this difficulty, the bulk of the available evidence indicates that macrophages are the primary source of PG among mononuclear cells (2-4). Even though macrophages synthesize greater quantities of PG and TX than lymphocytes, membrane AA content is approximately equal between these two heterogenous cell populations (3). The significance of this is unclear, but since lymphocytes release significant amounts of AA following activation, portions of this released AA may be taken up by macrophages and metabolized to TX or PG (3). This could provide a basis for lymphocyte regulation by macrophage PG synthesized from AA released from activated lymphocytes. But this type of self regulation is entirely speculative.

Regulation of lymphocyte function by exogenous PG has been extensively reported (2-5,44). Many of these reports indicate that PG inhibit immune reactivity in vitro (3,4,-45). Much of this evidence, however, was obtained in cultures in which exogenous PG were added, often to concentrations of 10^{-5}-10^{-4}M (3,45). If PG are physiologic effectors of lymphocyte functions, then effects should be seen at doses of PG in the range of 10^{-9}-10^{-7}M (3,46). But even within these physiological dose ranges the effects of PG

on lymphocyte responses are inconsistent, with results ranging from 75% inhibition of lymphocyte function to no effect at all (3,47,48). Since PG bind to serum proteins it has been suggested that the variation in reported results may reflect differences in experimental methods (3).

Besides adding PG to cultures, numerous investigators have added inhibitors of PG metabolism to block PG synthesis and test cell functions in the absence of PG (3,44). Indomethacin, which blocks cyclooxygenase, has been shown to significantly increase T cell blast formation (3,44). But indomethacin may affect other aspects of lymphocyte activation, making the results difficult to interpret on the basis of PG inhibition alone (3,49). Also, one recent report has shown that indomethacin augments mitogenesis only when suboptimal doses of the mitogen are used. When optimal doses are employed, indomethacin has no effect (3,50). Another aspect of this problem lies with the heterogeneity of T cells. It is now clear that T cells are a functionally diverse population of cells and that the various T cell subpopulations may differ in their sensitivity to PG (3).

The effect of PG on T cell subpopulations is particularly important in evaluating the evidence relating PG effects on antibody production. Several studies have shown that PGE_2 impairs in vitro antibody synthesis (3,4,51,52). There is now experimental evidence showing that if PGE_1 is present in the first 24 hours of the in vitro culture,

antibody production is increased at 5 days (3,53). Another study showed that increased PG production in vivo immediately following antigen injection correlated with higher antibody production at 5 days (3,51). One possible explanation for these results is that early in the antibody response PG may either enhance T cell helper function (T_H) or reduce T suppressor (T_S) function, thus enhancing antibody production.

Of central importance in discussing the effects of PG on T and B cell function is the role of the macrophage in PG production (2-4). As already mentioned, macrophages constitute the major PG producing immunocompetent cell (2-4). There is also evidence showing that macrophage PG production can influence the functional activity of T and B cells as well as macrophages themselves (2-4). Because of the requirement for macrophage participation in a number of lymphocyte responses it is possible that macrophages modulate T and B cell responses through PG synthesis.

In closing, sufficient data exist indicating that prostaglandins and other oxygenated metabolites of AA influence macrophage and lymphocyte function. With more information about the heterogeneity of macrophage and lymphocyte subpopulations, future studies should be able to discern the major prostaglandin producing and prostaglandin sensitive cell subpopulations and relate this information to the effects of prostaglandins on immune functions in

vivo. At this point it seems fairly clear that rather than initiate monocyte responses, PG and related lipids regulate or modulate these responses. This implies then, that EFA metabolites regulate immune functions. It is not possible, however, to tie together EFA intake, PG production, and regulation of the immune response. Part of the problem is the relationship between EFA intake and PG production is not well understood (54). And, as already discussed, the regulation of macrophage and lymphocyte subpopulations by PG and related lipids is just beginning to be understood.

EFA and the Lymphocyte Membrane

The lymphocyte plasma membrane is approximately 50% lipid by weight (17,55). About 70% of this lipid is phospholipid, with phosphotidlylcholine and phophotidyl-ethanolamine comprising almost 70% of total phospholipids (55). Studies by Ferber and Resch (55), indicate that the fatty acid composition of the resting lymphocyte membrane has a lower AA content than liver cells, indicating that the resting lymphocyte has a more rigid membrane structure. Following lymphocyte stimulation, however, the membrane fatty acid composition changes dramatically (55). Within the first hour of stimulation, enhanced incorporation of AA into membrane phospholipids occurs (55). This rapid and specific transfer of AA to the number two position of membrane phospholipids is

catalyzed by a plasma membrane enzyme, acyl Coa:lysolecithin aclytransferase (55). This enzyme exhibits a high affinity for AA, and its increased activity following lymphocyte activation changes the fatty acid composition of the activated lymphocyte to a more unsaturated, more fluid, state (55).

It has been postulated that a more fluid membrane may create a favorable environment for patching and capping of cross linked membrane receptors, thus enhancing lymphocyte activation (55). But currently the role of fluidity in receptor movement is unclear (17). Also, patching and capping are not obligatory events in lymphocyte activation, indicating that increased fluidity may not be essential to cell activation (17). But these studies do point out that regulation of membrane EFA in lymphocytes may differ from that of other cell types. This suggests that lymphocyte membrane EFA may perform different functional duties within the membrane than EFA in other cells. The effects of EFAD on lymphocyte membrane functions has not been studied. But in light of evidence indicating the importance of EFA in the lumphocyte membrane, this information could be important in understanding much of how EFA function in the lymphocyte membrane.

Lymphocyte Activation: Phospholipid Metabolism

Membrane lipids comprise the structural element of biological membranes. Since 70% of the lymphocyte membrane lipid component is phospholipid (55), changes in phospholipid metabolism are clearly important in membrane function. It is particularly significant, then, that one of the earliest events in lymphocyte activation is enhanced membrane phospholipid turnover (17,55-57). Although all reports are not in total agreement, enhanced turnover of phosphatidylcholine (17,55,56), and phosphatidylinositol (57) have been most often reported. Although the precise function of enhanced phospholipid metabolism is not known, it appears to be correlated with DNA synthesis in several experimental systems (17,55-57). In studies of ^{14}C-choline uptake into total lipids following Con A activation, it was shown that incorporation of the label into lipids: (1) occurred early following exposure to Con A (within 3 hours), (2) did not occur following exposure to a nonmitogenic lectin, (3) was proportional to mitogenic doses of the lectin, and (4) could be inhibited by a competitive inhibitor of Con A, (α-methyl-mannoside) (56).

As previously mentioned, membrane phospholipid fatty acid metabolism is also stimulated in activated lymphocytes (17,55). Results from several experiments indicate that phospholipid fatty acid turnover significantly affects membrane physical properties, and may affect the function

of membrane bound enzymes and receptors (58-59).

A direct link between enhanced phosphotidylinositol turnover and a membrane associated enzyme has recently been reported (59). In this report a Ca^{2+} dependent protein kinase (termed protein kinase C) was described. This enzyme was shown to be active when associated with membranes and dependent on diacylglycerol, unsaturated in the number 2 position (59). Although the physiologic substrates of protein kinase C are yet to be determined, phosphorylation of a number of cytoplasmic proteins accompanies lymphocyte activation (59). This may be a first step in unravelling the physiological significance of early phospholipid turnover in lymphocyte activation. Even without a complete definition of the role of protein kinase C in lymphocyte activation, the association of phospholipid turnover and enzyme activity suggests the importance of phospholipid metabolism during the lymphocyte activation process.

EFA and Lymphocyte Membrane Receptors, Enzymes, and Transport

While lipids are largely responsible for membrane structure, recent evidence suggests that specific membrane fatty acids may exist in "domains" (60), and protein lipid interaction within these "domains" may determine membrane protein function (60,61). This is particularly significant in lymphocytes since the initiating event in lymphocyte

activation is the binding of the external ligand to the lymphocyte membrane receptor (17). Several reports indicate that membrane receptors can be affected by alterations in membrane lipids (29,62-64). For example, it has been shown that EFA are required in the maintenance and induction of prolactin receptors (62). This implies that changes in membrane fatty acid composition resulting from EFAD directly affects the expression of a specific receptor. EFAD has also been shown to effect ^{125}I-Con A binding to purified liver plasma membranes, but the effects are opposite to those observed with prolactin receptors. In this case ^{125}I-Con A binding was increased in the plasma membrane preparations from animals fed the EFAD diet compared with preparations from animals fed a high fat diet (29). Total number of binding sites was increased, while binding affinity was unaffected by the diet. The increased binding appeared to be due to an increase in membrane surface glycoprotein content in the EFAD cells (29). This suggests that an increase in glycoprotein synthesis accompanies EFAD, but the physiologic significance of this is unknown.

EFAD induces changes in the number of Na^+, K^+-ATPase units (28) and enzyme activity (27). Since increased activity of Na^+, K^+-ATPase occurs early in lymphocyte activation, any external factor which affects its activity may affect lymphocyte activation. The bulk of available evidence indicates that mitogens bound to lymphocyte

membranes activate Na^+-K^+-ATPases, either by a direct interaction between the mitogen receptor complex and the enzyme, or indirectly by some general membrane perturbation induced by mitogen binding (17). Since oubain blocks all aspects of lymphocyte activation, regardless of stimulant used, it appears that Na^+, K^+-ATPase activity is vital to lymphocyte activation (17). At present it is unknown if the changes in Na^+,K^+-ATPase induced by EFAD affect the function of the enzyme in lymphocyte activation. It seems plausible though, that EFAD could alter lymphocyte Na^+, K^+-ATPase activity and this could contribute to reduced lymphocyte responses observed in EFAD animals.

Another series of membrane associated events occurring early in lymphocyte activation is enhanced metabolite transport (17). Enhancement of glucose transport during lymphocyte activation has been extensively reported (17). This increased transport appears to be due to increased transport Vmax values, but not Km, in mitogen activated lymphocytes (17). The extent to which mitogens increase glucose facilitated diffusion has been difficult to assess, however, because in most studies only a portion of the cells in the culture are activated by the mitogen. A large number of cells remain in the resting, or unstimulated state. Thus, total transport values reflect the sum of transport into these different populations of cells within an individual culture.

Whereas the role of increased membrane fluidity in receptor movement may be less than originally thought, it now appears that increased fluidity correlates with increased glucose transport (65-67). This has been demonstrated in intact cells (67) and purified membrane vesicles (65,66). With experimental evidence supporting a role for membrane fluidity in glucose transport, it seems possible that this transport may be affected by EFAD.

Along with enhanced glucose transport, enhanced amino acid transport also occurs early in lymphocyte transformation (68-71). One of the most thoroughly studied of the multiple amino acid transport systems is the so called "A" system for neutral amino acid transport (70,71). This system has been characterized using the labelled non-metabolizable amino acid analogue α-aminoisobutyric acid (AIB). It has been clearly established that enhanced AIB transport occurs early in lymphocyte activation and the total amount of AIB taken up by the lymphocyte is the sum of that transported by a saturable Na^+ dependent component, and that incorporated by Na^+ independent diffusion (67-71). In studies using Erhlich's ascites tumor cells grown in animals fed a diet high in saturated fat (the diet was EFAD (72)) it was shown that AIB transport is sensitive to dietary fat intake (73). The Km value for AIB uptake into tumor cells from mice fed the EFAD diet was almost double that of tumor cells from mice fed EFA sufficient diets (73). This shows that EFAD alters AIB transport into tumor

cells and indicates that this could occur in other cell types, including lymphocytes.

Lipoproteins and Immunity

Several recent reports have proposed that lipoproteins inhibit immune functions (74-81). Among the different lipoprotein classes, a subpopulation of LDL, termed LDL-In, was reported to contain the highest immunoinhibitory effect. In these studies human LDL-In was found to suppress mitogen responses and MLR of mouse spleen cells as well as in vivo plaque forming responses (74-76). Further experiments revealed that the biological activity of the LDL-In resided in the protein component, as the immunoregulatory functions remained after extraction with solvents (78,79). This indicated that the interaction of the LDL-In particle with the lymphocyte surface and the resulting impaired responses were independent of the lipid portion of the lipoprotein complex. One difficulty with these reports is that the LDL-In is a trivial name and it is not completely clear what the composition of LDL-In is. If the immunoregulatory properties of LDL-In reside solely in its protein moiety, then it should be fairly easy to assess this experimentally as the protein component of LDL is 98% apoprotein B (apo B) (82). This has recently been looked at in a study assessing the immunoregulatory properties of apoprotein B and

apoprotein E (apo E) (77). It was found that both apo B and apo E inhibited PHA stimulation of human peripheral lymphocytes. On a molar basis, apo E was approximately four times more effective in inhibiting PHA mitogenesis than apo B (77). It was suggested that apo E and apo B both bind to a common receptor on the lymphocyte surface ("immunoregulatory receptor") and this binding initiates the suppressive effect. Internalization of the lipoproteins, although it occurs, is not required for the immunosuppression as LDL linked to Sepharose also was immunosuppressive (77).

Another recent study has looked at the immunosuppressive effects of mouse lipoproteins (81). In general, the results agree with the previous reports, except these authors found that HDL was the predominant immunosuppressive lipoprotein, as opposed to LDL or an LDL subfraction.

In contrast to these reports, there is also evidence indicating that LDL enhance in vitro immune responses (83). Also, one report has found that oxidized LDL inhibits mitogenesis, but non-oxidized LDL has no inhibitory effect (84).

Another approach employed has been to use serum from animals fed different levels of dietary fat. Serum from rats fed a high PUFA diet was found to inhibit mitogen responses (85). Further experiments showed that the

lipoprotein fraction in general was responsible for the inhibition. This study also showed that spleen cells from rats fed a low fat diet (about 5% of calories from linoleic acid) responded better to mitogens than spleen cells from mice fed a high PUFA diet (about 40% of calories from stripped corn oil); and both groups of cells responded best to mitogens when cultures were performed in media supplemented with serum from rats fed the low fat diet (85). This suggests that the high PUFA diet may reduce immune functions, possibly by affecting lipoproteins. No evidence for shifts in the lipoprotein profiles between the two dietary treatments was presented, however. On the other hand, in short term studies, triglyceride rich serum from healthy individuals after consuming a high fat meal had no inhibitory effect on lymphocyte mitogenesis (86).

At present, the physiological significance of immunoregulation by lipoproteins is unclear. The bulk of the available evidence comes from studies in which exogenous lipoproteins have been added to in vitro cultures, with little discussion of how lipoproteins could regulate immune functions in vivo. Lipoproteins are not synthesized by cells of the immune system, nor is their synthesis induced by immunization. Thus, it is difficult to understand how lipoproteins could regulate immunity.

But dietary studies have shown that serum from animals fed moderate to high levels of PUFA impair mitogen responses

(85). This is puzzling as animals fed high PUFA diets are not immunosuppressed (12,13). In agreement with this, serum from patients with type IV or type V hyperlipoproteinemia inhibits mitogenesis, but there is no evidence that these individuals are immunodeficient (87). In the absence of any in vivo immunodeficiency it is difficult to understand the physiological significance of in vitro immunoregulation by serum from animals fed high PUFA diets. On the other hand numerous in vitro studies have shown that serum or lipoproteins from animals fed moderate to high levels of PUFA inhibit in vitro responses (74-81, 85). In particular, studies with lipoproteins have shown immunoinhibitory effects at physiological concentrations, indicating that under normal conditions lipoproteins may affect lymphocyte function (88). Studies correlating dietary PUFA intake, lipoprotein profiles, and immunity are needed to put this complicated problem in order.

EFA and Disease

Epidemiological evidence indicates that societies that consume low fiber diets relatively high in fat, particularly of animal origin, (Western type diet) have a higher incidence of colon and breast cancer than societies consuming a high fiber diet lower in animal products and fat (89-91). On the other hand, lower incidences of stomach and liver cancer are seen in societies consuming the

Western type diet (90). Immigrant studies show that migrating peoples assuming the customs and eating habits of their new home also assume the types of cancer characteristic of that area of the world (90,91). All this points to a relationship between dietary habits and certain cancers. But the picture is complicated by more than eating habits. Other factors such as smoking, environmental pollution, alcohol consumption, caffeine consumption, and stress have been implicated (90,91). Continued efforts in both epidemiological and experimental investigations are needed to define the relationship between dietary patterns and susceptibility to certain cancers.

Experimental studies, however, do indicate a relationship between dietary fat and certain types of tumors. Over the past 15 years a number of reports have appeared demonstrating a relationship between dietary PUFA intake and increased appearance and growth of chemically induced and transplanted mammary tumors in rodents (92-95). Also, in the past several years a relationship between dietary fat and the induction of chemically induced colon cancer has been described (96). It has been proposed that dietary fat acts as a tumor promoter, and that this promotion may involve the immune system (94), presumably by affecting tumor sureillance.

Although dietary PUFA are correlated with the development of chemically induced and transplanted mammary tumors, it appears that tumor growth is approximately equal

regardless of the PUFA level fed, as long as the EFA requirements are met (93,95). With chemically induced colon cancer, a high fat diet similar to that of high risk populations correlates with an increased incidence of chemically induced tumors (89,96). While these studies indicate a link between dietary fat intake and certain types of tumors, there is no evidence that the immune system is involved.

Current progress in tumor immunology indicates that the concept of a thymus-dependent tumor surveillance mechanism is undergoing revision (97). A better understanding of anti-tumor surveillance is necessary to determine if the immune system is implicated in the relationship between dietary fat and cancer.

In an earlier review several diseases were associated with dietary EFA intake (6). In one of these diseases, multiple sclerosis (MS), some additional work has been reported in the past few years. The basis of the relationship between dietary EFA intake, immunity, and MS lies with the observation that MS patients respond to immunosuppressive agents (98,99), and they have low serum EFA levels (100, 101). These observations, together with those from animal models of MS, indicated that EFA may reduce the symptoms of MS (102-104). Results from clinical studies, however, showed that patients receiving EFA supplements (3-8 g/day linoleic acid) faired no better than their

non-supplemented free-living fellow patients (105-107). Other reports previously discussed (6), have also been inconclusive. Thus, the link between EFA and MS remains to be proven.

Other topics previously reviewed include EFA, immunity, and transplantation, and cardiovascular disease. Few reports further exploring links between dietary EFA, the immune response, and these two conditions have appeared in the literature in recent years.

Also discussed were reports looking at the direct relationship between EFAD and resistance to infection (6). Despite the possibility that there may be a relationship between the EFAD of TPN patients and their increased susceptibility to infection, the most recent report of a direct relationship between EFAD and increased susceptibility to infection was in 1948 (108).

EXPERIMENTAL

Effects of Essential Fatty Acid Deficiency, and Diets High in Polyunsaturated Fatty Acids, on Delayed Type Hypersensitivity in Mice

Introduction

Several recent reports have proposed that PUFA regulate, or modulate, the immune response by specifically impairing T cell responses (9-11,109,110). For example, mice receiving daily injections of PUFA demonstrated impaired allograft rejection (9-11) and cytoxicity (9-11); while addition of PUFA dissolved in ethanol to lymphocyte cultures reduced in vitro T cell mitogenesis (111). In only a few cases, however, has the relationship between dietary PUFA and in vivo T cell responses been evaluated. But these results are difficult to interpret due to improper diet formulation (7), and lack of quantitative presentation of immune response data (8). Effects of dietary saturated fatty acids and PUFA on in vitro T cell transformation have also been reported, however the results are contradictory (110,112).

I showed earlier that in vivo antibody-mediated immunity was not impaired in mice fed a high PUFA diet (50%

corn oil (CO)). Reduced antibody responses were observed only in mice fed an essential fatty acid deficient (EFAD) diet 0% (CO) (12). The assay used in these experiments (Jerne plaque assay) measured the number of B cell progeny secreting antibody in response to immunization with sheep red bloom cells (SRBC). Since it is well known that this B cell response requires the active participation of T helper (T_H) cells (113), my data indicated that at least one aspect of in vivo T cell function (T_H cell function) was not impaired in mice fed a high PUFA diet.

Before using in vitro assays to assess mechanisms whereby dietary PUFA may affect T cell function, I wished to further determine if the dietary manipulation affected T cell responses in vivo. Taking this approach, I assessed the T cell dependent delayed-type hypersensitivity (DTH) response as described by Vadas, et al (114).

The DTH response is an inflammatory reaction resulting from a complex interaction between sensitized T cells (T_D), which bear the Ly 1 surface alloantigen, B cells, and macrophages (114-116). Although both T_D cells and macrophages are involved in this response, macrophage participation depends upon the specific activation of sensitized T_D cells (114-116). In the experiments reported here, I tested the DTH response of mice fed the EFAD (0% CO), control (13% CO), or high PUFA (50% CO) diets.

Materials and Methods

Animals and Diets

Male A/J mice 21 days of age were housed, five per cage, in plastic solid bottom cages in a temperature (24 1°), light (12 hours light per day), and humidity controlled room. Diets and water were provided ad libitum. Three diets were formulated on an equal energy basis, providing 0, 13 or 50% of dietary energy from corn oil, and 24% of dietary energy from casein. Complete composition of the diets is in Table 1.

Experiment 1. To determine effects of the EFAD and high PUFA diets on DTH, mice were fed the diets for 70 days. To measure DTH, a modification of the method of Vadas et al. (114) was used. Body weights were also assessed.

Experiment 2. Two aspects of the relationship between EFAD and T_D cell function were examined: 1) the length of time required to observe losses in the DTH response after introduction of the EFAD diet, and 2) the length of time required to reverse these losses by refeeding the control diet. Mice were fed the EFAD diet (0% CO) or the control diet (13% CO) for 77 days. A third group of mice was fed the EFAD diet for 63 days and then switched to the control diet for 7 or 14 days. In vivo DTH was assessed at 7 day intervals from 17 to 77 days.

Table 1. Composition of Diets.

	Diet		
Ingredient	EFAD	Control	High PUFA
Basal mix, g[1]	35	35	35
Glucose, g	65	54	20
Corn oil, g	0	5	20
% Energy from corn oil	0	13	50

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

Determination of DTH. DTH was measured by a modification of the method of Vadas, et al (114). Mice were sensitized to dinitrofluorobenzene (DNFB) by spreading 50 ul of a 2% solution of DNFB onto shaved areas (2 cm diameter) of their backs and abdomens. Five days later 10 ul of a 1% DNFB solution was applied to the right ear, and a nonspecific agent (turpentine) was applied to the left (control ear. Ten hours after the second administration of DNFB, mice were injected with 2 uCi of ^{125}I-iodo-deoxyuridine (5 Ci/mmol, Amersham). Twenty-six hours after the second administration of DNFB mice were killed and both ears were removed and counted in a gamma counter. Results were calculated as follows:

$$\text{Stimulation index} = \frac{\text{cpm (test ear)} - \text{cpm (background)}}{\text{cpm (control ear)} - \text{cpm (background)}}$$

Data analysis. All data were treated statistically by either the student's t-test, or one way analysis of variance with treatment differences assessed by Tukey's test (117).

Results

Experiment 1. As I had observed previously (12), body weights of mice fed the EFAD diet for 70 days were reduced compared to weights of mice in the control and high PUFA

groups (Table 2). Food intake was not measured, but in an earlier study (12) mice fed the high PUFA and control diets consumed slightly, but not significantly, more than mice fed the EFAD diet. DTH was impaired in mice fed the EFAD diet (Table 2), but the response of mice fed the high PUFA diet did not differ from the controls. These findings are in close agreement with my previous report on the humoral response (12), and demonstrate that when relatively high levels of PUFA are fed, no inhibitory effects on in vivo immune functions are seen. On the other hand, when mice consume an EFAD diet, in vivo immunity is impaired.

Experiment 2. To examine the temporal relationship between consumption of an EFAD diet and in vivo T cell function I monitored the DTH response of mice over a 77 day period (Figure 2). After 42 days, a significant reduction in DTH was observed in mice fed the EFAD diet. This reduction remained fairly constant at about 75% of the control response for the remainder of the experiment (77 days).

To determine if the inhibitory effects of the EFAD diet on DTH were reversible, two groups of mice fed the EFAD diet for 63 days were switched to the control diet (13% CO). After only 7 days of refeeding, the DTH response was completely restored (Figure), and even exceeded

Table 2. Body Weight and Delayed Type Hypersensitivity Response of Mice Fed the Experimental Diets for 10 Weeks.[1]

	% Dietary Energy From Corn Oil		
	EFAD	Control	High PUFA
Body weight[2] (g)	23.0±0.7[a]	27.5±1.2[b]	29.8±1.4[b]
SI[3]	1.73±0.13[a]	2.51±0.20[b]	2.33±0.13[b]

[1]Means for 10 mice. Numbers with different superscripts are significantly different ($p < 0.05$).

[2]Mean initial body weights were 9.6 grams.

[3]$$SI = \frac{\text{cpm } ^{125}\text{I-iododeoxyuridine incorporated into stimulated ear-cpm (background}}{\text{cpm } ^{125}\text{I-iododeoxyuridine incorporated into control ear-cpm (background)}}$$

See Materials and Methods for a description of the method.

Figure 2. Delayed-type hypersensitivity response of mice fed an essential fatty acid deficient diet (0% corn oil). Response expressed as a percentage of control (13% corn oil) response. Each point represents the mean of 10 mice. A = significantly different from control ($p < 0.05$); B = significantly different from refed ($p < 0.05$).

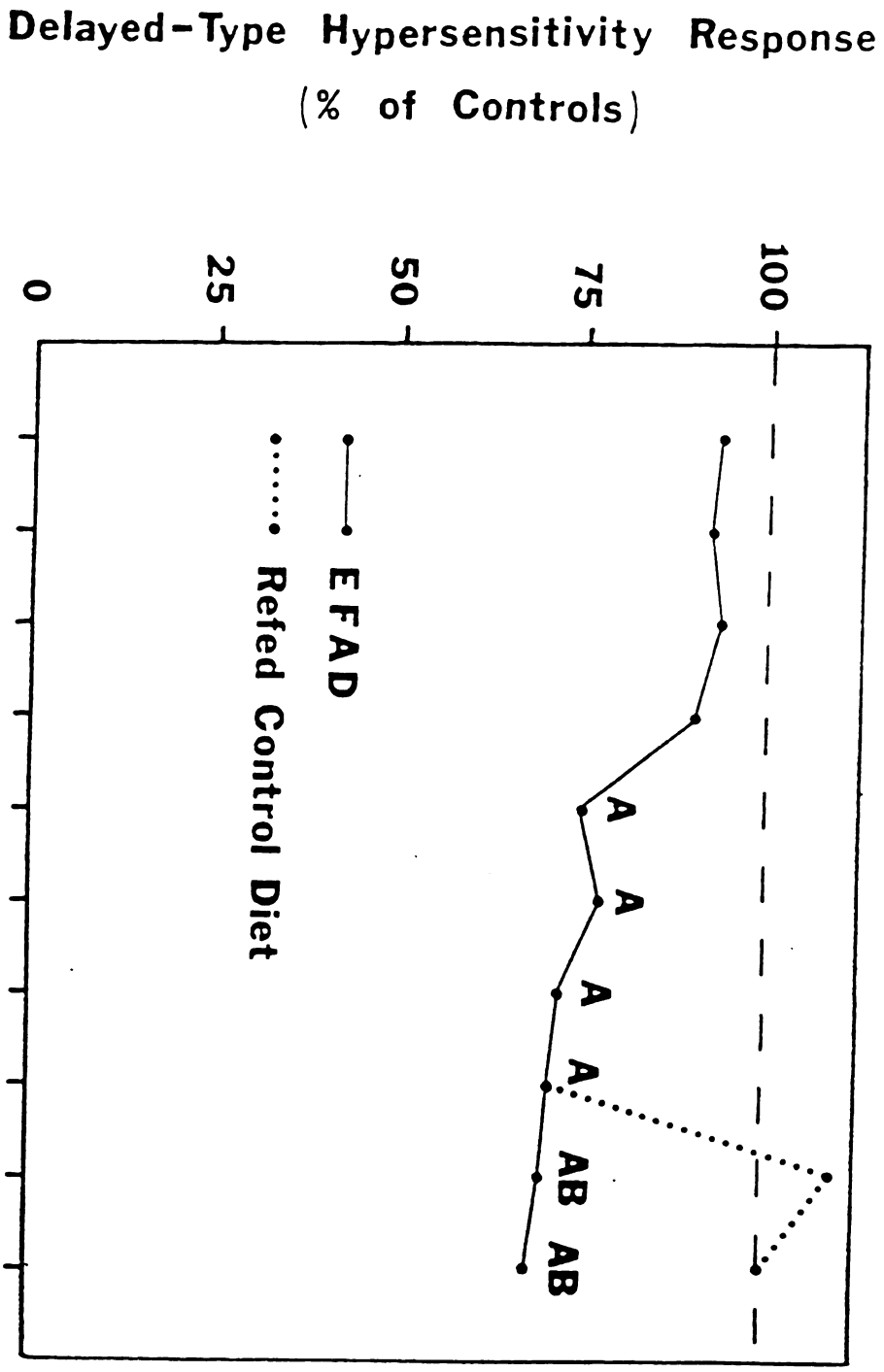
Delayed-Type Hypersensitivity Response
(% of Controls)
100
75
50
25
0
A
A
A
A
AB
AB
EFAD
Refed Control Diet
14 21 28 35 42 49 56 63 70 77
Time (Days)

Figure 2.

the control response by about 15%. The DTH response after two weeks of refeeding was approximately equal to the control response. At both time points the DTH response of mice remaining on the EFAD diet was significantly reduced compared to the response of the control or the refed groups. Thus, the deleterious effects of the EFAD diet on DTH are reversible, with recovery occurring rapidly (within 7 days) after refeeding a diet containing EFAs.

Discussion

This report extends previous observations (12) related to the influence of EFAD and high PUFA diets on in vivo immune responses. In both studies, consumption of an EFAD diet (0% CO) depressed in vivo responses, whereas consumption of a high PUFA diet (50% CO) for an extended period of time (70 days) had no adverse effects on in vivo responses. These findings, particularly those related to PUFA and in vivo T cell responses, do not agree with the conclusions of others (9-11). Several reasons for these differences are possible.

Others reported a consistent pattern of immunosuppression when mice fed stock diets were injected daily with 10 ul of PUFA (linoleic or arachidonic acid) (9-11). Based on a daily intake of 12 kcal/day (12), and assuming 90% absorption of PUFA (118), mice fed my control diet (13%

of energy from CO (60% linoleic acid)) consumed about 94 mg linoleic acid/day and mice fed the high PUFA diet received about 360 mg linoleic acid/day. If the daily intake of stock diet fed to mice injected with PUFA contained approximately 100 mg PUFA, then injection of 10 mg PUFA increased intake only 10%. Since mice fed my high PUFA diet increased PUFA intake by nearly 300% without a reduction in in vivo T cell function, it is difficult to attribute the immunosuppression observed after injection of PUFA to PUFA alone. Although two reports suggest that dietary PUFA suppress in vivo T cell responses, interpretation of these studies is difficult because in one case the experimental diets were grossly different in their nutrient density (7), and in the other, quantitative assessments of T cell responses were not used (8).

Conflicting reports have appeared on effects of high PUFA diets on T cell mitogenesis in vitro (110,112). Erickson, et al (110), reported that a high PUFA diet (approximately 44% of energy from safflower oil) impaired mitogenic responses of spleen cell from young mice to Con A. However, Ossman, et al (112), presented data showing that Con A responses of spleen cells from adult mice increase with increasing amounts of corn oil in the diet. Another in vitro approach has been to add PUFA, or PUFA bound to albumin, directly to cell cultures. Addition of PUFA has been shown to decrease (119), increase (120), or have no

effect on mitogen responses (121). Thus, these data have not helped clarify affects of high PUFA intake on T cell function.

It has also been proposed that PUFA "deficiency" is immunopotentiating (10) because graft rejection was faster in mice fed an autoclaved (supposedly to specifically destroy PUFA double bonds) diet. I never found the EFAD diet (which could be termed "PUFA deficient") to be immunopotentiating in any in vivo study. On the contrary, in vivo immunity has been consistently depressed in mice fed the EFAD diet. Furthermore, since autoclaving would be expected to destroy several water soluble vitamins, in addition to PUFA double bonds, it is difficult to see how this diet could enhance the immune response.

I found that both DTH and antibody-mediated responses were reduced within 21-28 days after feeding the EFAD diet. This reduced capacity to respond in vivo plateaued at approximately 60-75% of the control response, where it remained for the duration of both experiments (70-77 days). Even though I have not studied the effect of EFAD past 77 days, it appears that despite an extended period of EFA deficiency, the immune system maintains some capacity to respond in vivo. Also, after 63 days of consuming the EFAD diet, the DTH response of mice switched to the control diet was rapidly (7 days) restored to control levels. This is consistent with my previous findings (12), and indicates

that EFAD-induced alterations in immune functions are rapidly reversed by feeding a diet contaiing EFAs.

Results presented here, and in my previous report (12), indicate that feeding mice high levels of PUFA has no adverse effects on in vivo immune functions. On the other hand, in both reports, in vivo immune functions were reduced in mice fed the EFAD diet. Both in vivo antibody-mediated immunity and DTH responses result from complex interactions among several cell types, including T and B lymphocytes and macrophages (113,114). Because of the complexity of these responses, it is not possible from available data to identify the precise cell population affected by the EFAD diet. Further studies are needed to determine the cell type and mechanism(s) responsible for the effects of EFAD on immune functions.

Finally, my results do not agree with previous reports proposing that PUFA decrease immunity (10,110) and "PUFA deficiency" increase immunity (10,110). However, differences in experimental approach probably account for the disparity in results. Therefore, PUFA may impair lymphocyte responses under certain experimental conditions such as subcutaneous injection (9-11), or direct addition to cell cultures (111). But when PUFA are consumed in the diet, no adverse effects on immune functions are seen.

Essential Fatty Acid Deficiency and Lymphocyte Activation I. Effects on Proliferative Responses and Phospholipid Metabolism

Introduction

As structural components of biological membranes essential fatty acids (EFA) influence membrane fluidity and membrane bound enzymes and receptors (1,55). In addition, EFA are precursors for cellular synthesis of prostaglandins and related lipids (1,2,33). In immunocompetent cells, incorporation of EFA into membrane phospholipids and the production of a wide range of EFA metabolites has been amply documented (2-5). Despite this understanding of the biochemistry of EFA metabolism in immunocompetent cells, information about the relationship between dietary EFA and in vivo immune functions is generally lacking.

Several studies have proposed that polyunsaturated fatty acids (PUFA), particularly EFA, inhibit in vivo immune functions (9-11). Much of the evidence supporting this proposal however, comes from studies in which graft rejection was prolonged in mice injected subcutaneously with purified PUFA (linoleic acid,18:2; or arachidonic acid 20:4) (10,11). In other studies graft rejection was also delayed in mice receiving purified PUFA by stomach tube (10). Based on a general estimation of the daily energy intake of adult mice consuming a stock diet and the experimental protocols used, the injected PUFA represented an

increase in daily PUFA intake of about 10%, and the gavage an increase in PUFA intake of about 50%. Since these protocols differ significantly from normal feeding, the question arose as to whether or not similar effects would occur if mice were fed elevated levels of PUFA, or EFA, in a controlled dietary study.

I investigated this question and showed that in vivo plaque forming and delayed type hypersensitivity responses of mice fed a high PUFA diet (50% of energy from corn oil) was equal to responses of mice fed an isocaloric control diet (13% of energy from corn oil). In comparing this dietary protocol with the injection and gavage experiments it is noteworthy that the daily PUFA intake of mice fed the high PUFA diet exceeded the intake of control fed mice by nearly 300% without adversely affecting in vivo immune functions. These observations make it difficult to attribute the immunoinhibition reported in the PUFA injection studies to PUFA alone.

In addition to proposing that PUFA inhibit immune functions it has also been reported that "PUFA deficiency" enhances immunity (10,11,110). Since animals do not have a requirement for PUFA in general, but do require a subset of PUFA, called EFA, it is difficult to test this proposal. However, I have looked at the effects of an EFAD diet on in vivo plaque forming and delayed type hypersensitivity responses in mice. Rather than being enhanced, the responses

of mice fed the EFAD diet was markedly depressed compared with the control and high PUFA groups (12,13).

The purpose of the present investigation was to further examine the relationship between dietary EFA and immune functions using cell culture. Although in vitro studies provide controlled conditions for manipulating experimental variables, culture conditions can influence cell responses in vitro (122-124). Thus, one of the difficulties in interpreting in vitro results is that as the time in culture lengthens, cell responses begin to reflect the culture environment more than in vivo dietary effects. Recognizing this, I assessed a series of events in lymphocyte activation ranging from those occurring within the first few hours of stimulation to those occurring after a few days in culture. In addition to looking at different time points during lymphocyte activation in vitro, I altered the in vitro environment to study the responses of cells from EFAD mice in the presence and absence of EFA.

Materials and Methods

Animals and Diets. Male A/J mice 21±3 days of age were housed in plastic cages, five/cage in a temperature (24±1^{o}), light (12 hours per day), and humidity controlled room. Diets and water were provided ad libitum. Composition of the diets is presented in Table 1.

T and B Cell Counts. After a 70 day feeding period spleen and thymus cell suspensions were prepared in DMEH, (M. A. Bioproducts, Walkersville, MD) and mononuclear cells isolated over Lympholyte M (Accurate Chemical & Scientific Corporation, Westbury, NY). For B cell counts, 100 ul of mononuclear cells (10^7/ml) were incubated with 50 ul of Immunobeads (BioRad, Richmond, CA) coated with rabbit antimouse IgG. After 60 minutes at room temperature, the mixture was centrifuged at 150xg and gently resuspended. Ten ul of 0.15% toluidine blue was added and rosettes (B cells) were read as positive if 3 or more beads were attached to a cell. For T cell counts, mononuclear cells (10^7/ml) plus rabbit antimouse T cell antiserum (Accurate Chemical and Scientific Corp., Westbury, NY) were incubated at 4^{o} for 60 minutes. The mixture was then centrifuged, resuspended in medium plus rabbit complement (Accurate Chemical & Scientific Corporation, Westbury, NY), and incubated at 37^{o} for an additional 60 minutes. Fifty ul of 1% trypan blue was added and dead cells (T cells) were counted. Results are expressed at % B or T cells. T & B cell percentages were calculated on the basis of total mononuclear cells.

Mitogen Proliferation Experiments. After a 56-70 day feeding period, mice were killed and spleen cell suspensions were prepared under sterile conditions in RPMI 1640 or DMEH

with 25 mM HEPES supplemented with 1 mM glutamine, and 0.2 ml of penicillin streptomycin (M. A. Bioproducts, Walkersville, MD) and 5 x 10^{-5} M 2-mercaptoethanol (Sigma, St. Louis, MO). FCS (Grand Island Biological, Madison, WI), bovine serum albumin (BSA) (Sigma, St. Louis, MO) or dl FCS protein prepared by the method of Rothblatt et al. (125), were added to the culture media in selected experiments. Spleen cell suspensions (2.5 x 10^6 nucleated cells/ml) were cultured in a total volume of 0.2 ml in round-bottom microculture plates (Linbro, Hamden, Cn) in a humidified Mishel-Dutton atmosphere (10% CO_2, 7% O_2, 83% N_2). Both unstimulated (no mitogen) and stimulated (containing mitogen) culture wells were prepared in triplicate. Viability was determined by trypan blue exclusion at the beginning and end of each experiment. Optimal mitogenic doses of Con A, PHA, PWM, and LPS (all from Sigma, St. Louis, MO) were used in all experiments. At 48 hours all cultures were pulsed with 0.1 uCi of ^{3}H-thymidine and harvested 12 hours later on glass microfiber filters (Whatman, Clifton, NJ) using a sampling manifold (Millipore, Bedford, MA). Radioactivity associated with TCA-precipitated DNA was counted in a liquid scintillation counter. Results are expressed as cpm ^{3}H-thymidine in DNA/culture well.

Mixed Lymphocyte Reaction (MLR). MLR was performed by a modification of the method of Meo (126). Briefly, spleen cell suspensions from A/J mice (responder cells) fed the three experimental diets were prepared at a concentration of 2.5×10^6 nucleated cells/ml. Stimulator cells were spleen cells from male C57/bl mice. Stimulator cells (7.5×10^6/ml) were treated with mitomycin C (50 ug/ml) (Sigma, St. Louis, MO) for 30 minutes at 37^o, washed 3 times with cold culture medium, counted, and resuspended, at a final concentration of 5×10^6/ml. One hundred microliters of both responder (2.5×10^5) (A/J) spleen cells and stimulator (5×10^5) (C57/bl) spleen cells were then added together in microtiter plates and cultured for 96 hours in a humidified Mishell-Dutton atmosphere. Control responses were assessed by incubating 2.5×10^5 A/J spleen cells without the addition of the stimulator cells. To check the effect of the addition of Mitomycin C treated cells on ^3H thymidine uptake an additional control was run in which Mitomycin C treated A/J spleen cells (5×10^5) were incubated with 2.5×10^5 A/J responder cells. Since ^3H thymidine uptake into these cultures did not differ from uptake into cultures containing unstimulated A/J spleen cells alone, these data were omitted. At 96 hours each culture well was pulsed with 0.1 uCi of ^3H-thymidine and 12 hours later cells were harvested, DNA precipitated, radioactivity counted and results expressed as previously described for the mitogen experiments.

Incorporation of Phospholipid Precursors into Total Lipids. Spleens were removed from mice fed the experimental diets and single cell suspensions were prepared in RPMI 1640 plus 0.15% dl-FCS protein. Spleen cell suspensions were then layered over Lympholyte-M and splenic mononuclear cells recovered from the interface between media and the Lympholyte-M after centrifugation. Mononuclear cells were then washed twice with cold culture medium, counted, and viability determined by trypan blue exclusion. Viability was similar for all treatment groups and exceeded 95%.

Mononuclear cells (10 x 10^6/ml) were cultured in triplicate in a humidified Mishell-Dutton atmosphere at a final volume of 0.2 ml in round-bottom microculture plates. Optimal mitogenic doses of Con A and 0.5 uCi of ^{14}C-choline or 1.0 uCi of ^{3}H-inositol (Amersham, Arlington Heights, IL) were added at the initiation of the cultures. Unstimulated cultures received label only. After the specified time in culture lymphocytes were removed and washed with cold culture medium. Cell pellets were then extracted with chloroform:methanol:10% trichloroacetic acid (8:4:1) and the chloroform layer was then dried under air and counted in a liquid scintillation counter. In preliminary experiments lipids were separated by thin layer chromatography. Approximately 90% of the ^{14}C-choline and ^{3}H-inositol recovered in total lipids was associated with phospholipids. This indicated that the incorporation of

the labelled phospholipid precursors into total lipids predominantly reflected uptake into phospholipids and in subsequent experiments separation of lipid classes was omitted.

Results in these experiments were expressed as: (Δcpm)

Δcpm = (cpm incorporated into lipids of Con A activated wells-background)

- (cpm incorporated into lipids of unstimulated wells-background)

Addition of Fatty Acids (FA) to Bovine Serum Albumin (BSA). A modification of the method of Spector and Hoak (127) was used. Briefly, 20 mg of 20:4 or 18:0 (99% pure, Sigma, St. Louis) was dissolved in 8 ml hexane and added to beakers containing 600 mg of Celite (Jons Manville, Madison, WI). The mixture was dried under nitrogen and vortexed. Twelve ml of Spector's balanced salt solution (127) containing 200 mg of BSA (essentially fatty acid-free, Sigma, St. Louis, MO) was then added to the Celite:FA particles and the solution was mixed for 30 minutes at room temperature, spun at 2^{o} at 15,000 x g for 10 minutes, and the supernatant recovered. Protein was analyzed by the method of Lowry, (128) and FA by the method of Mosinger (129).

Preparation of dl-FCS. Dl-FCS was prepared by the method of Rothblatt et al, (16). Twenty ml of FCS was

slowly added to 200 ml of ice-cold acetone-ethanol (1:1) while gently swirling. The mixture was allowed to stand overnight at 0^{o}. The delipidated protein was collected on Whatman #1 filter paper by suction over a Buchner funnel. The protein was washed with 50 ml of ethyl ether. After 15 minutes the dried powder was scraped from the filter paper and stored dessicated until use.

Results

Experiment 1. In this experiment I looked at whether or not EFAD directly alters either the total number of mononuclear cells or the percentage of T and B cells in two lymphoid tissues, the thymus and the spleen. I found no differences across the three dietary treatments in either the total number of mononuclear cells or the percentage of T and B cells in either tissue (Table 3). This indicates that consumption of neither the EFAD, nor the high PUFA diet results in loss of mononuclear cells or lymphocyte populations. The immunoinhibitory effects of the EFAD diet, therefore, appear to be directed at events occurring during the lymphocyte activation process.

Experiment 2. Spleen cells from mice fed the experimental diets were stimulated with optimal doses of Con A, PHA, PWM, and LPS, in cultures supplemented with 5% FCS. Although not statistically different, mitogenic responses of spleen cells from mice fed the EFAD diet were

Table 3. Mononuclear Cell Counts, and Percentage of T and B Cells in Lymphoid Organs of Mice.

Diet	Total # of Mononuclear Cells	% B Cells	% T Cells
	Spleen		
EFAD	3.65×10^7	30.5±1.7	32.5±0.6
Control	3.47×10^7	32.5±2.8	34.5±1.4
High PUFA	3.50×10^7	33.4±3.2	34.0±0.9
	Thymus		
EFAD	1.03×10^7	3.8	96
Control	1.04×10^7	2.5	95
High PUFA	0.98×10^7	3.2	95

[1]Mean ± SEM of 4 mice per group. Mice 21±3 days of age were fed the diets for 70 days.

[2]T and B cells as percentage of total mononuclear cells.

consistently higher than the other two dietary treatment groups (Figure 3).

One explanation for the slightly higher mitogenic responses of spleen cells from mice fed the EFAD diet was that they may have "repaired" during the culture period with exposure to the small amount of EFA contained in FCS. To further test the importance of in vitro "repair" I supplemented FCS containing cultures with BSA:20:4. For comparison, BSA:18:0, a SFA, was added to parallel cultures. Optimal stimulation of mitogen proliferation was observed in both EFAD and control spleen cells when cultures were supplemented with BSA:FA conjugates at a molar ratio of 1:2 (BSA:FA) and at a final concentration of 0.1 umoles/ml: 0.2 umoles/ml (data not shown). Addition of BSA conjugated fatty acids (20:4 or 18:0) did not increase ^{3}H-thymidine incorporation into spleen cells from EFAD or control mice above levels observed in cultures containing FCS alone (Figure 4). Next, spleen cells were cultured in lipid free media. Even under these conditions ^{3}H-thymidine incorporation into spleen cells from EFAD mice was comparable to values in spleen cells from control mice (Figure 3). Addition of BSA:20:4 to the lipid free cultures improved Con A responses of both EFAD and control spleen cells, but not to levels obtained in FCS containing cultures. BSA:18:0 had no effect on Con A responses. In general, viability was similar across the treatments

Figure 3. ^{3}H-thymidine incorporation into TCA precipitated DNA of mitogen activated spleen cells from mice fed an EFAD, control or high PUFA diet. Spleen cells (2.5×10^{5}) were cultured for 60 hours in DMEH plus 5% FCS and optimal doses of each mitogen. ^{3}H-thymidine (0.1 uCi) was present during the final 12 hours. The basal (unstimulated) ^{3}H-thymidine incorporation was 299±36 (EFAD), 232±23 (control) and 269±37 cpm/well (high PUFA). All results represent the mean ± SEM of triplicate observations from each of 10 mice/treatment after a 56-70 day feeding period which was initiated when the mice were 21±3 days of age.

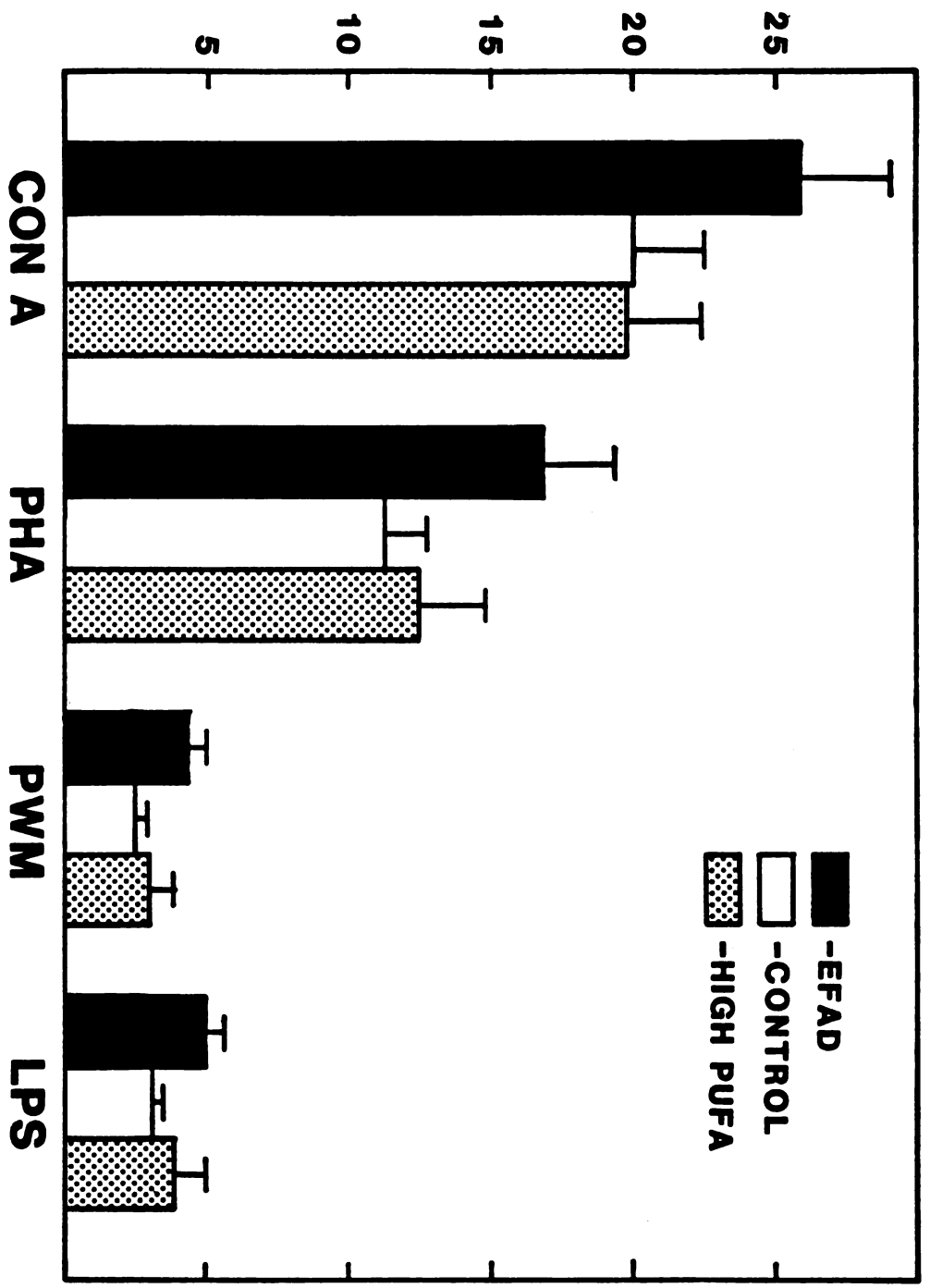
cpm ^{3}H-thymidine in DNA x 10^{-3}/well
5
10
15
20
25
CON A
PHA
PWM
LPS
-EFAD
-CONTROL
-HIGH PUFA

Figure 3.

Figure 4. Effects of the addition of BSA:FA conjugates on ^{3}H thymidine incorporation into TCA precipitated DNA of Con A activated spleen cells from mice fed an EFAD, control, or high PUFA diet. Spleen cells (2.5×10^{5}) from each treatment were incubated for 60 hours in 5% FCS (upper panel) or serum free (lower panel) cultures with the addition of BSA: 20:4 or BSA: 18:0 at a molar ratio of 2:1. ^{3}H-thymidine (0.1 uCi) was present for the final 12 hours. Data represent the mean ± SEM of duplicate or triplicate observations on each of 8-10 mice per treatment after a 56-70 day feeding period. Mice were 21±3 days of age at onset of the feeding period.

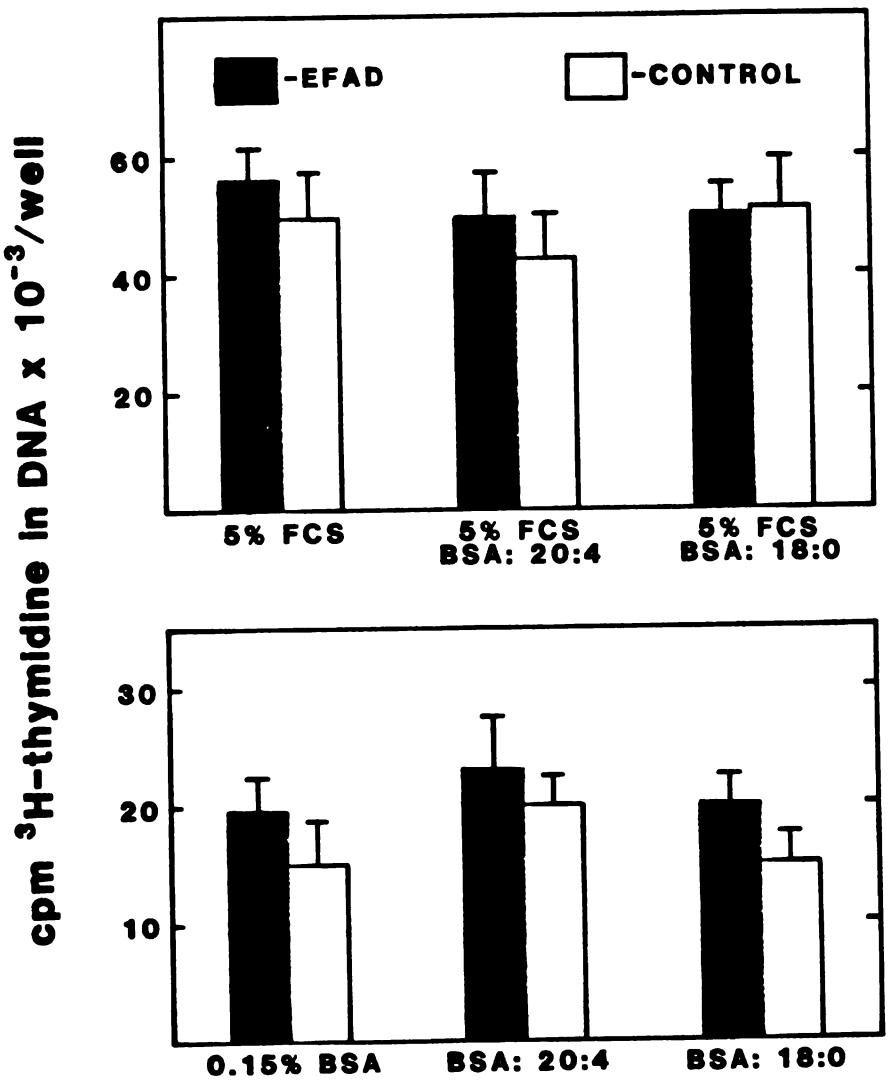
-EFAD
-CONTROL
cpm ^{3}H-thymidine in DNA x 10^{-3}/well
60
40
20
5% FCS
5% FCS
BSA: 20:4
5% FCS
BSA: 18:0
30
20
10
0.15% BSA
BSA: 20:4
BSA: 18:0

Figure 4.

and reduced in cultures that did not contain FCS. Viability was reduced from approximately 75% in FCS cultures to about 40% in BSA containing cultures. This reduced viability in BSA containing cultures probably contributed to the reduced responses in these cultures.

Experiment 3. Results from Experiment 2 indicated that in vitro mitogenic responses from mice fed the experimental diets were similar; whereas earlier reports had shown that in vivo immune functions were reduced in mice fed the EFAD diet compared with mice fed the control or high PUFA diets. In this experiment I assessed the proliferative response of spleen cells from mice fed the experimental diets to allogeneic spleen cells in a one-way mixed lymphocyte reaction (MLR). Mitomycin C treated spleen cells from C57/bl mice served as the allogeneic stimulator cells. The results (Table 4) showed no differences across the three treatments for the one-way MLR.

Experiment 4. The lack of agreement between the in vitro results from Experiment 2 and 3 and previous in vivo studies (12,13) suggested the further possibility that the culture conditions may have influenced the in vitro results. Therefore, two variables were changed. First, in an effort to reduce time in culture I switched from ^{3}H-thymidine incorporation (which requires several days for expression)

Table 4. One Way Mixed-Lymphocyte Reactions (MLR)[1] ^{3}H-Thymidine Uptake cpm/well.

Diet	Responders	Responders & Stimulators
EFAD	1254±324	4417±1141
Control	1044±293	4148±901
High PUFA	1142±354	3739±937

[1]Spleen cells (2.5×10^{5}) from A/J mice fed the three experimental diets were incubated with 5.0×10^{5} mitomycin-C treated spleen cells from C57/Bl mice. Cells were incubated for 108 hours, with 0.1 uCi of ^{3}H-thymidine presen for the final 12 hours. Data represent the mean ± SEM of duplicate observations from each of 10 mice/treatment.

to phospholipid metabolism (which occurs within a few hours of lymphocyte activation) (55-57). Second, to study lymphocyte activation under EFA-free conditions I used a serum-free, lipid-free culture supplement, dl-FCS.

In preliminary experiments I found that ^{3}H-thymidine uptake in response to Con A was 40% higher in dl-FCS than in BSA-containing cultures, and background stimulation in dl-FCS was reduced 75% compared with BSA cultures. Since optimal ^{3}H-thymidine uptake in response to Con A and minimal background values were obtained using 0.15% dl-FCS, this level was used in all experiments discussed.

First, I assessed Con A induced ^{14}C-choline uptake into cellular lipids after 240 and 480 minutes in culture (Figure 5). After 240 minutes in FCS containing cultures, no differences in ^{14}C-choline uptake were seen across the treatments. However, after only 240 minutes in lipid free culture, Con A induced ^{14}C-choline uptake into total lipids of spleen cells from mice fed the EFAD diet was significantly reduced (approximately 60%) compared with uptake into cells from mice fed the control or high PUFA diet. If cultures were extended to 480 minutes, no treatment differences were seen in either the lipid free of FCS containing cultures.

Similarly, Con A induced ^{3}H-inositol uptake into cell lipids was assessed (Figure 5). In agreement with the results for choline no differences were seen across the

Figure 5. Incorporation of phospholipid precursors into total lipids of Con A activated lymphocytes from mice fed the EFAD, control or high PUFA diet cultured in media containing either FCS or delipidated FCS (dl-FCS). Splenic mononuclear cells (1 x 10^6) were incubated with optimal doses of Con A, or without mitogen (unstimulated) plus 0.5 uCi of ^{14}C-choline or 1.0 uCi of ^{3}H-inositol. Harvested lymphocytes were extracted with 8:4:1 (chloroform:methanol: 10% TCA) and radioactivity associated with the chloroform layer was counted. Radioactivity specifically associated with Con A activation (Δcpm) was determined by subtracting the counts incorporated into total lipids from unstimulated cells from counts incorporated into the total lipids Con A activated cells after background was subtracted from both. Data represent the mean ± SEM of duplicate observations on each of 8-10 mice/treatment after a 56-70 day feeding period begun when the mice were 21±3 days of age. (*) = significantly different (pl 0.05) than the control or high PUFA groups.

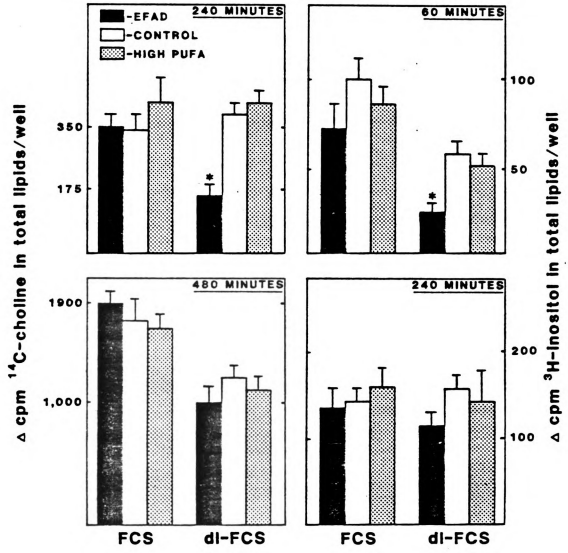
-EFAD
-CONTROL
-HIGH PUFA
240 MINUTES
60 MINUTES
480 MINUTES
240 MINUTES
350
175
100
50
1900
1,000
200
100
*
*
Δ cpm ^{14}C-choline in total lipids/well
Δ cpm ^{3}H-inositol in total lipids/well
FCS
dl-FCS
FCS
dl-FCS

Figure 5.

treatments in FCS containing cultures at 60 minutes. However, after only 60 minutes in the lipid-free culture, Con A induced ^{3}H-inositol uptake was significantly reduced in lymphocytes from mice fed the EFAD diet compared with the other groups.

Discussion

I previously showed that in vivo plaque forming responses and delayed type hypersensitivity were impaired in mice fed an EFAD diet compared with mice fed a control or high PUFA diet (12,13). In this section, I investigated the effects of these dietary treatments on lymphocyte activation in vitro.

EFAD could impair in vivo immune responses by altering total mononuclear cells, or the proportions of lymphocyte populations. In this experiment I determined total mononuclear cell numbers and percentages of T and B cells in two lymphoid tissues (thymus and spleen) of unimmunized mice fed the three experimental diets. Values were similar across the three treatments for all measurements (Table 3). The absence of any dietary effects on mononuclear cell numbers, or T and B cell proportions, suggests that EFAD affects in vivo immunity by altering the functional capacity of existing cells, rather than by eliminating specific cell populations. However, the assays used to

determine T and B cell counts do not rule out the possibility that EFAD could alter T and B cell subpopulations, and in this manner influence in vivo immunity.

The presence or absence of certain T and B cell subpopulations can be estimated, to some degree, by their response to specific mitogens. T helper cells (T_H), for example, bear the Ly 1 surface antigen and constitute the major responding T cell subpopulation to Con A and in the MLR (130,131). T cells bearing Ly 1, 2, 3, are PHA responsive (130). Also, LPS appears to preferentially stimulate immature B cells (132). Since proliferative responses to all these mitogens were similar across the three dietary treatments under standard culture conditions, this suggested that the diets did not alter lymphocyte subpopulations.

Previous studies had shown that when EFAD mice were switched to a diet containing EFA, in vivo plaque forming and delayed type hypersensitivity responses returned to normal, and even overshot control responses (12,13).

Since the proliferative responses of spleen cells from mice fed the EFAD diet were equal to, or slightly higher than the responses from the other two treatments this suggested the possibility that the EFAD spleen cells may have "repaired" in vitro in the presence of the small amount of EFA in FCS. But further supplementation of FCS cultures with EFA (20:4) had no effect on Con A responses

of either EFAD or control spleen cells. This suggested that the small amount of EFA provided by FCS was sufficient for lymphocyte proliferation regardless of the dietary treatment. Others have reported conflicting results; supplementation of serum containing cultures with additional fatty acids either free or bound to BSA has been shown to increase (120), decrease (119), or have no effect (121) on lymphocyte proliferation. Variations in serum, or ratio of albumin to fatty acid, may be contributing factors in the lack of agreement among reports (121).

Although overall proliferative responses of both treatments were reduced by more than 50% in lipid free cultures compared with FCS cultures, the response of EFAD spleen cells remained slightly higher than the controls. Thus, even in the absence of any exogenous source of EFA, the EFAD spleen cells retained the capacity to respond to Con A as well as, or slightly better than, control spleen cells. Con A responses of both treatments improved by about 20% with the addition of 20:4 to lipid free cultures. This is in general agreement with previous reports indicating that adding PUFA, particularly EFA, improves cell growth and proliferation compared with cultures containing only BSA alone, or BSA plus a SFA (133). But again, there was no evidence of treatment differences following the addition of 20:4 to the cultures.

These experiments demonstrate that spleen cell viability

and proliferative responses to mitogens and MLR were not impaired by EFAD. Several reasons could account for this. Lymphocyte proliferation may not require EFA, or, when EFA are present in the culture, the amount required for proliferation may not be altered by dietary EFA status.

Another possibility is that during EFAD lymphocyte responses are suppressed, either by the presence of a suppressive factor, or by the absence of some activating factor derived from EFA, and this suppressive effect is released when cells are cultured and assayed in vitro. It is also possible that the manner in which EFAD impairs in vivo immune functions may not be detected by in vitro proliferation assays. Another consideration is the extended culture time required for assessment of lymphocyte proliferation. Since it is well established that cultured cells incorporate exogenous lipids over time in culture, it seems probable that proliferation experiments requiring several days in culture provide little insight into the functional properties of EFAD lymphocytes as they exist in vivo.

To minimize affects associated with long term cultures, I investigated the influence of the dietary treatments on lymphocyte phospholipid metabolism, an important membrane associated event occurring within the first few hours of lymphocyte activation (55-57). When these short term cultures contained FCS, no treatment differences

were detected. However, in lipid free cultures, there was a transient reduction in Con A induced uptake of ^{14}C-choline and ^{3}H-inositol into EFAD spleen cells compared with the other two treatments. The loss of dietary treatment effects over time in culture strongly supports the notion that long term culture experiments reflect in vitro conditions rather than in vivo dietary effects.

This appears to be true for EFAD, but may not be the case for all nutrient deficiencies. Decreased Con A proliferative responses have been shown in experimental studies of zinc deficiency (134,135), vitamin E deficiency (136), and protein calorie malnutrition (137). In both zinc deficiency and protein calorie malnutrition however, decreased Con A responses have been associated with shifts in the lymphocyte populations induced by the deficiency (135,137).

Since EFAD does not appear to shift lymphocyte populations or subpopulations my results suggest that impaired early phospholipid metabolism in spleen cells from mice fed the EFAD diet results from a membrane-associated defect induced by EFAD. This defect may not be detected in proliferation experiments because of extensive lipid rearrangements occurring over several days in culture. On the other hand, phospholipid metabolism in Con A activated EFAD cells in FCS cultures was similar to the control and high PUFA groups. A likely explanation for this is that some "repair" of the EFAD cells occurred in the

presence of EFA in FCS.

Although it seems highly probable that the lack of EFA in the lipid free cultures contributed to the impaired early phospholipid metabolism in Con A activated EFAD spleen cells, other explanations are possible. For example, the delipidation procedure may have extracted or denatured growth factors in FCS. It is possible that EFAD spleen cells may be more sensitive to the loss of growth factors, or possibily the production of toxic compounds by the delipidation procedure. But since phospholipid metabolism in the Con A activated EFAD spleen cells was similar to the other two treatments at a later time point, these alternate explanations for the early impairment seem unlikely. The loss of treatment differences with time in culture is consistent with the proposal that long term in vitro responses reflect culture conditions to a far greater extent than in vivo dietary effects, at least in the case of EFAD.

Recent studies indicate that enhanced phospholipid metabolism is a precommitment event in the cell cycle which is proportional to Con A concentrations, even very high levels (100-200 ug/ml) (56,57). Unlike phospholipid metabolism, DNA synthesis is optimally stimulated within a very narrow, relatively low dose range of Con A (2.5-10 ug/ml). To facilitate comparisons between early precommitment events and DNA synthesis, all phospholipid

cultures discussed here contained doses of Con A optimal for DNA synthesis. Within this dose range early phospholipid metabolism was significantly delayed in EFAD spleen cells, but DNA synthesis was not. Whether or not a similar early impairment follows antigen activation in vivo is not known. But previous studies following the development of plaque forming cells (pfc) after in vivo immunization with SRBC indicated a significant delay in the sequence of appearance of IgM and IgG pfc, as well as peak pfc response, in mice fed the EFAD diet (12). The delayed appearance of pfc would be consistent with an EFAD-induced impairment early in lymphocyte activation. Also, since EFAD alters membrane structural lipids (1), it seems likely that in addition to phospholipid metabolism, other membrane-associated events may be affected by EFAD. Presumably, the combined effects of these EFAD induced defects impairs lymphocyte function.

Finally, others have proposed that PUFA impair the immune response. In the present report, the responses of lymphocytes from mice fed the high PUFA diet equalled the response of the control group in all assays measured. This supports my previous findings which showed that in vivo immune functions were not impaired in mice fed an isocaloric, high PUFA diet (12,13).

Essential Fatty Acids and Lymphocyte Activation

II. Effects on Neutral Amino Acid Transport

Introduction

Because of the importance of EFA in the lipid component of biological membranes, EFAD could impair lymphocyte responses by altering the lymphocyte membrane structure (1). Alterations in structure could affect lymphocyte responses by influencing a number of membrane-associated events that occur early in lymphocyte activation. One of these events, phospholipid metabolism, has already proven to be sensitive to EFAD, as shown in the previous section. Another early event in lymphocyte activation is the increased influx of metabolities (17), including amino acids (17,68-71). In this section I examined neutral amino acid transport using α-aminoisobutyric acid (AIB). This transport system increases in activity early in lymphocyte activation (68-71) and correlates with DNA synthesis (69). AIB transport in ascites tumor cells is also sensitive to dietary fat intake (73).

I assessed the capacity of resting (unstimulated) and Con A activated lymphocytes from mice fed the experimental diets to take up ^{14}C-AIB. I found that Con A activated EFAD lymphocytes were defective in their capacity to transport AIB at low substrate concentrations (0.01 mM). At 0.01 mM AIB, reduced AIB uptake by lymphocytes from

mice fed the EFAD diet elevated Km, but not Vmax values for initial Na^+-dependent transport. Taken together with results from the phospholipid experiments, this indicates that lymphocytes from mice fed an EFAD diet have defects in several membrane-associated events prominent in the first few hours of lymphocyte activation. These defects may contribute to the overall impairment in the immune response which accompanies EFAD (12,13).

Materials and Methods

Animals and Diets. Male A/J mice, 21±3 days of age, were housed in plastic cages, in a temperature (24 ± 1^{o}), light (12 hours per day), and humidity controlled room. Mice were fed ad libitum one of the three experimental diets described in Table 1 for 70-84 days.

Preparation and Preliminary Incubation of Splenic Mononuclear Cell Suspensions. Red cells, dead cells, and granulocytes were removed from spleen cell suspensions by centrifugation over Lympholyte M. Viability of the mononuclear cell suspension collected from the culture medium/gradient interface always exceeded 95% and did not differ across the three dietary treatments.

Before assessing AIB uptake, mononuclear cells (10^7/ml) were preincubated with or without optimal mitogenic

doses of Con A for 240 minutes at 37^{o} in a humidified atmosphere of 7% CO_2, 10% O_2, and 83% N_2. All cell preparations and preliminary incubations were conducted aseptically in RPMI 1640 plus 25 mM HEPES, 2 mM glutamine, and 0.15% delipidated fetal calf serum protein (dl-FCS). Dl-FCS was prepared by the method of Rothblatt et al, (125).

Time Course of ^{14}C-AIB Accumulation. After the 240 minute preincubation, cells were washed twice with cold culture medium, and resuspended in 0.2 ml of Earle's Balanced Salt Solution containing 25 mM HEPES and 0.1 uCi of ^{14}C-AIB (Amersham, Arlington Heights, IL). At specified times, uptake was stopped by the addition of 1.0 ml of cold phosphate buffered saline containing 5 mM AIB (PBS-AIB). Cells were then collected on glass microfiber filters using a sampling manifold, and washed twice with 10 ml of cold PBS-AIB. After the filters were dried, scintillation cocktail was added and accumulated radioactivity determined by counting in a liquid scintillation counter. To correct for extracellular trapped radioactivity, 0.1 uCi of ^{14}C-AIB was added to cell suspensions kept at 4^{o} and counts associated with these tubes subtracted from experimental values. Results are expressed as total accumulation of AIB in picomoles/10^6 mononuclear cells.

AIB Initial Rate Measurements. Total cellular AIB uptake includes AIB entering the cell by a saturable Na^+ dependent transport system and by simple diffusion (70,138). The contribution of each of these components to total AIB uptake can be estimated by performing parallel experiments in isosmolar Na^+ containing and Na^+ free media. The active Na^+ dependent component of AIB uptake is calculated by subtracting AIB uptake in Na^+ free media from AIB uptake in Na^+ containing media.

To assess total AIB uptake incubation media (IM-1) was prepared as previously described by Finklestein and Adelberg (139). IM-1 contained 150 mM NaCl, 1.0 mM $CaCl_2 \cdot 2H_2O$, 5.0 mM $K_2HPO_4 \cdot 3H_2O$, 1.2 mM $MgSO_4 \cdot 7H_2O$, 0.3 mM H_3PO_4, and 5.6 mM D-glucose. The pH was 7.4. Following the 240 minute pre-incubation mononuclear cell suspensions were washed twice with cold IM-1 and resuspended in 0.2 ml of IM-1. After a five minute incubation in a 37^o shaking water bath, various concentrations of unlabelled AIB plus 0.1 uCi of ^{14}C-AIB were added to each tube. After 120 seconds 1.0 ml of ice cold IM-1 plus 5 mM AIB was added to each tube as the tubes were placed in ice. Spleen cells were then centrifuged in a microfuge (Eppendorf, Brinkman Instruments, Westbury, NY) for 30 seconds over a 20% Percoll gradient (Pharmacia, Piscataway, NJ). Cell pellets were then washed twice with ice cold IM-1 containing 5 mM AIB, resuspended in saline, and transferred to scintillation vials

to which 15 ml of ACS scintillation cocktail (Amersham, Arlington Heights, IL) was added. Radioactivity was determined by counting in a liquid scintillation counter. Results are expressed as picomoles AIB uptake/10^6 cells/minute.

To determine the Na^+ independent or diffusion component of AIB uptake IM-2 was formulated in an identical manner to IM-1, except 150 mM choline chloride replaced NaCl. Experiments were performed in an identical fashion as described for total AIB uptake, except in all instances IM-1 was replaced with IM-2. The active Na^+ dependent component of AIB transport was then calculated by subtracting values obtained in incubations using IM-2 from those using IM-1. Results are expressed as AIB uptake in picomoles/10^6 cells/minute. By using the Percoll gradient and washing the pellets as described, negligible counts were detected in the pellets as extracellular or trapped counts.

Data Analysis. Data were analyzed statistically by one way analysis of variance with treatment differences determined by Tukey's test (117). Results relating substrate concentrations to initial velocity were determined by least squares analysis with graphic transformation shown by plotting v vs v/[AIB].

Results

Time Course of AIB Uptake. Enhanced neutral amino acid transport occurs early in the process of lymphocyte activation (68-71) and is sensitive to dietary fat saturation (73), at least in ascites tumor cells (73). Figure 6 shows the time course of the AIB (0.01 mM) accumulation into splenic lymphocytes from mice fed the EFAD, control or high PUFA diets. AIB accumulation into unstimulated lymphocytes was characterized by a rapid initial uptake in the first 120 minutes, followed by little additional uptake between 120 and 240 minutes. Although AIB accumulation into unstimulated lymphocytes from EFAD mice was slightly less than for lymphocytes from the control or high PUFA groups, no statistical differences were evident.

AIB accumulation into Con A stimulated lymphocytes, however, was significantly reduced in the EFAD group compared with the control or high PUFA groups (Figure 6). Since viability was similar across the three treatments (approximately 80% of the original cell number), the reduced uptake into the EFAD cells was not due to decreased cell numbers in the EFAD cultures.

AIB Uptake Kinetics. Next I investigated initial AIB uptake rates in Con A activated lymphocytes from mice fed the three diets. After 240 minutes in lipid-free medium containing optimal mitogenic doses of Con A, total, Na^+-dependent, and Na^+-independent AIB uptake, as well as

Figure 6. Time course of AIB uptake. Resting (---) and Con A activated (——) (2.5 ug/ml) splenic lymphocytes (10 x 10^6/ml) were incubated in a total volume of 0.2 ml with 0.10 uCi of ^{14}C-AIB in RPMI 1640 plus 0.15% dl-FCS. At the time points indicated, incubations were stopped by placing the cultures in an ice bath and adding 1.0 ml of ice cold PBS-AIB. Cellular accumulation of AIB was assessed after collecting cells on glass microfiber filters and washing with PBS-AIB. Data represent the mean ± SEM of six experiments.

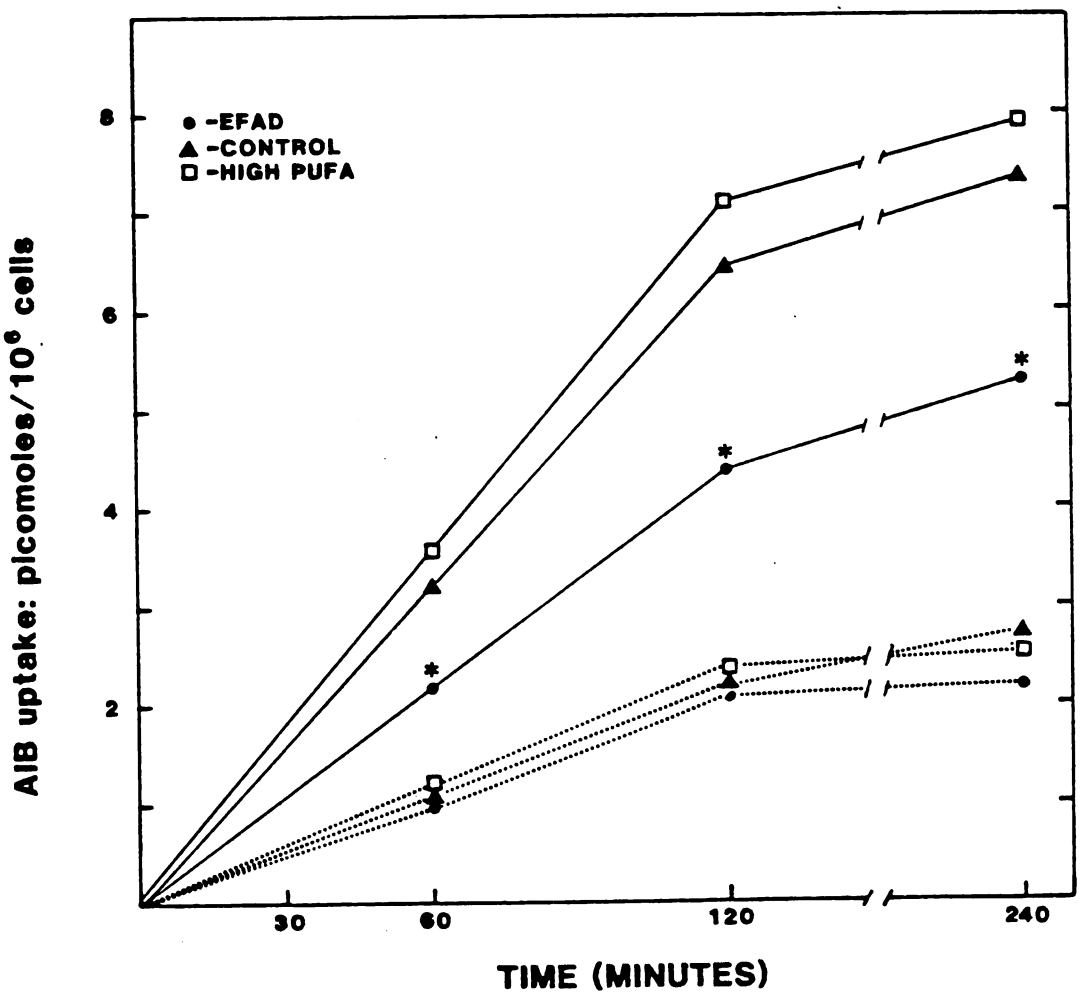
● -EFAD
▲ -CONTROL
□ -HIGH PUFA
AIB uptake: picomoles/10⁶ cells
8
6
4
2
30
60
120
240
TIME (MINUTES)

Figure 6.

initial transport rates of Na^+-dependent uptake were assessed. A 120 second incubation period was used to measure initial uptake rates.

Over the doses of AIB used (0.01-4 mM), I observed a characteristic biphasic curve for AIB uptake (Figure 7A). Total uptake into EFAD lymphocytes was significantly reduced compared with the other two treatments at the .01 mM AIB concentration (Figure 7A insert). At this level of AIB, which was identical to the AIB concentration used in the time course experiments, the capacity of activated EFAD lymphocytes to transport AIB was impaired. However, no differences occurred among the treatments for AIB uptake at AIB concentrations from 0.035-4 mM.

As these experiments were run in Na^+ buffer (Materials and Methods) parallel experiments were also run in Na^+-free buffer (Materials and Methods) to determine the Na^+-independent, or diffusion component of total AIB uptake. When this component is subtracted from total AIB accumulation, values for Na^+ dependent AIB transport are obtained (Figure 7B). As previously shown with total AIB uptake, Na^+ dependent AIB transport was significantly lower in the EFAD cells at AIB concentration of 0.01 mM. (Figure 7B (insert)). No differences were seen across the three treatments in Na^+ dependent AIB transport at AIB concentrations ranging from 0.035-4.0 mM.

The kinetics of AIB transport were assessed in lymphocytes

Figure 7A. Total AIB uptake. Following a 240 minute pre-incubation (Materials and Methods) Con A activated splenic lymphocytes (10 x 10^6 ml) were washed and resuspended in 200 ul of IM-1 (Materials and Methods) plus 0.10 uCi of ^{14}C-AIB and various amounts of cold AIB. After a 120 second incubation in a 37^o water bath, incubations were stopped by adding 1.0 ml of ice-cold PBS-AIB and placing the cultures on ice. Cell-associated radioactivity was determined after cells were pelleted by centrifugation through a 20% Percoll gradient. Data represent the mean ± SEM of five experiments.

7B. Na^+ dependent AIB uptake. To determine Na^+ dependent AIB uptake parallel experiments were performed as described for total AIB uptake, except Na^+ free medium (IM-2) was used. The difference between values obtained in IM-1 and IM-2 are considered to represent Na^+ dependent AIB uptake. Data represent the mean ± SEM of five experiments.

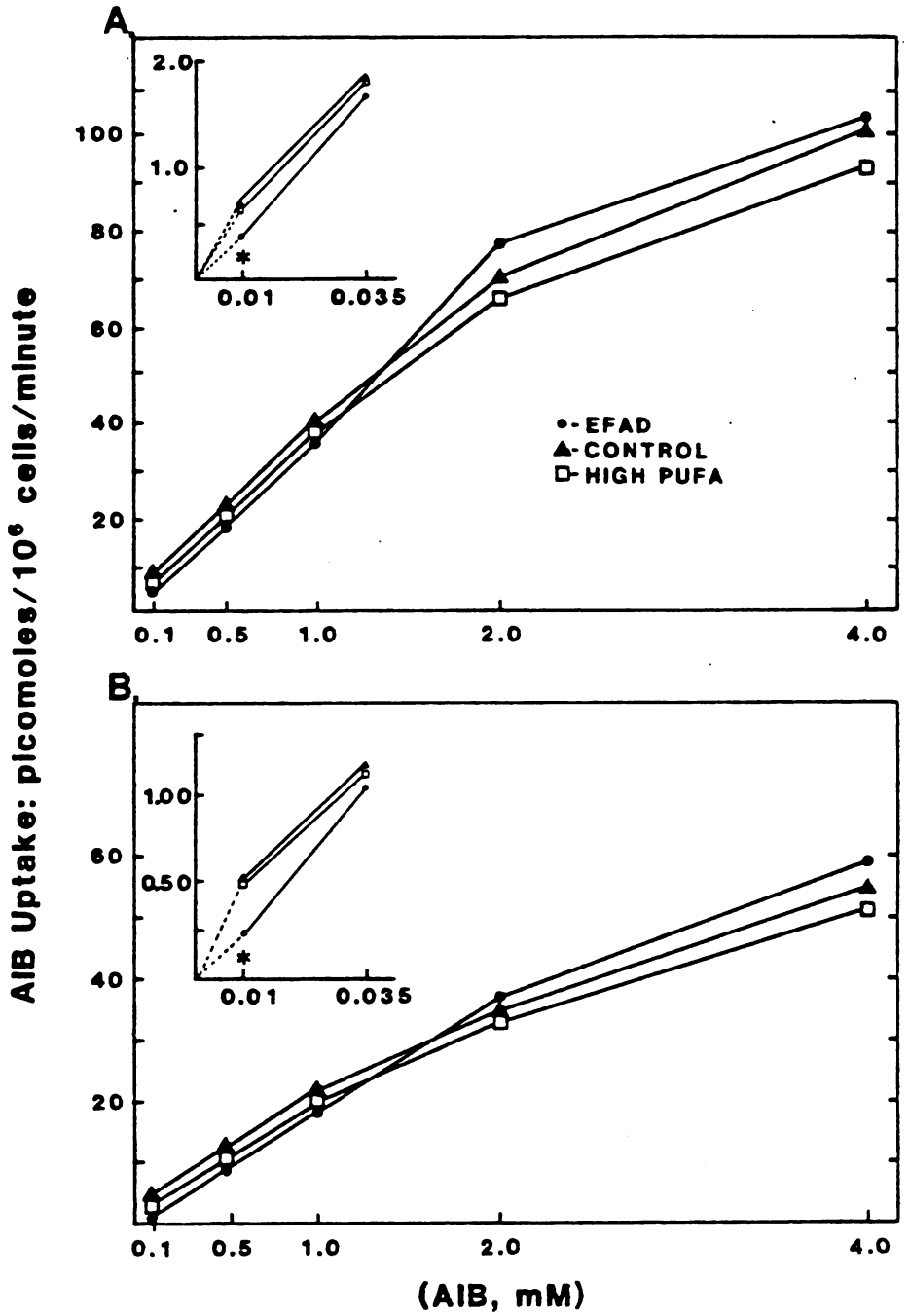
A
B
AIB Uptake: picomoles/10⁶ cells/minute
(AIB, mM)
•-EFAD
▲-CONTROL
□-HIGH PUFA
0.1
0.5
1.0
2.0
4.0
0.01
0.035
20
40
60
80
100
1.0
2.0
0.50
1.00

Figure 7.

from mice fed the three experimental diets. Results are presented in Figure 8, with initial velocity (v) of Na^+ dependent transport plotted against v/[AIB]. Lines derived from least squares analysis of experimental values for each treatment were plotted, and the Km and Vmax values estimated graphically. From this analysis the Km value for the EFAD lymphocytes was almost twice as high as the values for the control or high PUFA groups (2.88 mM vs 1.54 and 1.66 mM), and the Vmax value for the EFAD group was approximately 50% higher than the other two treatments (92 pmol vs. 63 and 61 pmol 10^6 cells/min).

To facilitate statistical analysis, Km and Vmax values obtained from each experiment (n = 5) were derived and mean values plus standard errors calculated. The results (Table 5) are essentially the same as those obtained graphically (Figure 8). The Km value for AIB transport in the EFAD lymphocytes were significantly greater than the Km value for the control or high PUFA groups. However, the Vmax value for the EFAD group, while remaining almost 50% higher than the Vmax values for the other two groups, did not differ statistically from these two treatments ($p < .05$).

Figure 8. Initial rate kinetics of Na^+ dependent AIB uptake into Con A activated splenic lymphocytes. Suspensions of Con A activated splenic lymphocytes (10×10^6/ml) were incubated for 120 seconds in various concentrations of unlabelled AIB plus 0.1 uCi ^{14}C-AIB. Data obtained are plotted by the Eadie-Hofstee method and represent mean values from five experiments. Best fit lines were obtained by linear regression of the experimental values with the following r^2 values; EFAD = .608, control = .675, and high PUFA = .855.

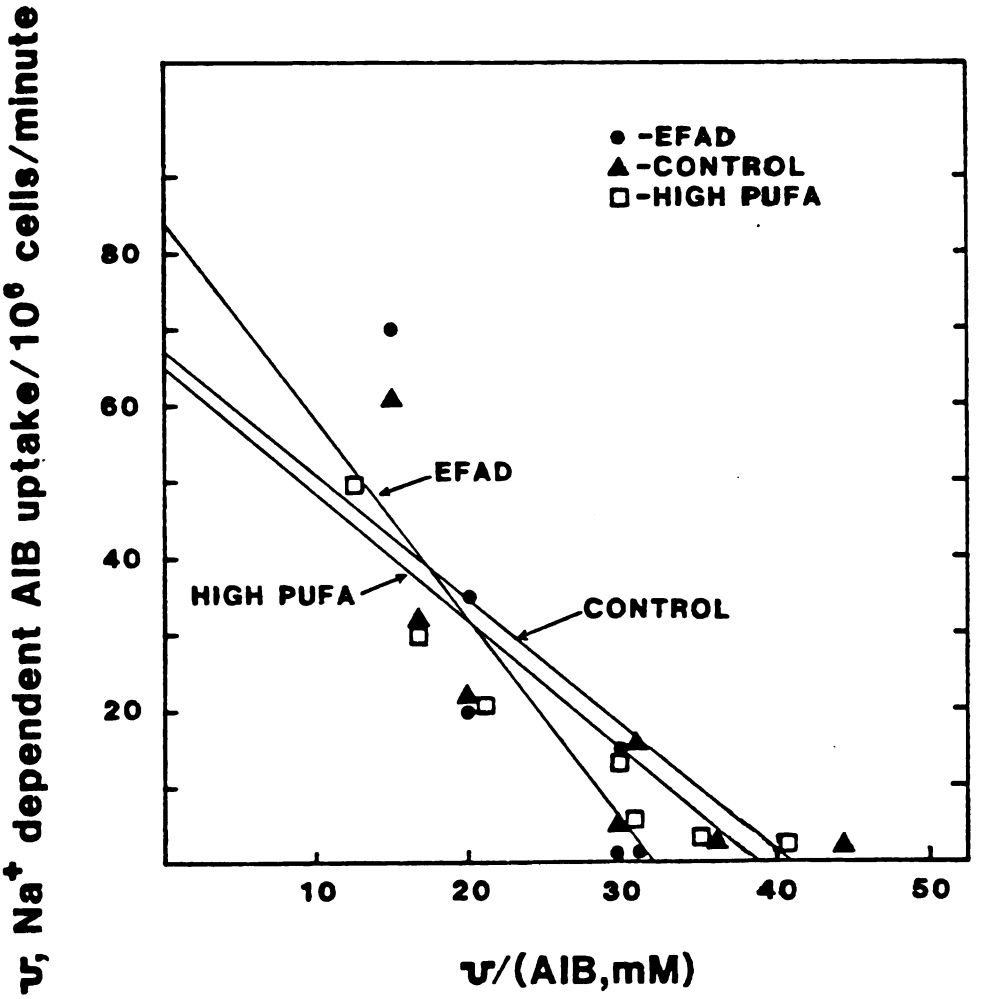
● -EFAD
▲ -CONTROL
□ -HIGH PUFA
EFAD
HIGH PUFA
CONTROL
80
60
40
20
10
20
30
40
50
υ, Na+ dependent AIB uptake/10⁶ cells/minute
υ/(AIB,mM)

Figure 8.

Table 5. Km and Vmax Values for Na^+ Dependent AIB Uptake Into Con A Activated Lymphocytes.[1]

Diet	Km (mMol)	Vmax (pmol/10^6 cells/min)
EFAD	2.36 ± 0.36^a	83 ± 14^a
Control	1.54 ± 0.17^b	64 ± 10^a
High PUFA	1.43 ± 0.13^b	55 ± 9^a

[1]Data represent mean ± SEM of 5 experiments. Numbers in the same column with different subscripts are significantly different ($p < 0.05$).

Discussion

The purpose of the experiments in this section was to further study the effects of dietary EFA and EFAD on early events in lymphocyte activation. I found that when extracellular AIB concentrations were low (0.01 mM), AIB uptake into Con A activated EFAD lymphocytes was impaired. This was true for AIB uptake over an extended time course (0-240 minutes), for total AIB uptake under conditions approaching initial rates (120 seconds), and for the Na^+ dependent component of initial AIB uptake. AIB uptake (0.01 mM) into Con A-activated EFAD lymphocytes was significantly reduced compared with uptake into Con A-activated lymphocytes from the control or high PUFA groups; but, again, only when AIB concentrations were low (0.01 mM). At higher AIB concentrations (0.035-4.0 mM) AIB uptake was similar across the three treatments. As a result, the Km value for Na^+ dependent AIB transport into Con A-activated EFAD lymphocytes was almost twice the Km values for the control and high PUFA groups, but Vmax values did not differ. This finding is in close agreement with the results obtained by Kaduce, et al, (73) in their study of AIB transport into Ehrlich's ascites tumor cells grown in mice fed diets differing in fat saturation. They found significantly elevated Km, but not Vmax, values for Na^+ dependent AIB transport into tumors grown in mice fed a diet high in hydrogenated coconut oil (EFAD).

The importance of the high affinity (low Km) component of neutral amino acid transport in mitogen activated lymphocytes has recently been demonstrated by Borghetti, et al. (71). They showed that two independent saturable components systems ACS and A) contribute to the entry of neutral amino acids into mitogen activated lymphocytes, and enhanced activity of the high affinity component (system ACS) is important for subsequent steps in lymphocyte activation. Given the importance of the high affinity component of neutral amino acid transport in lymphocyte activation, it seems plausible that its disruption in mitogen activated EFAD lymphocytes may alter their response capacity. This could occur in several ways.

The most direct way that EFAD could influence membrane transport would be through alterations in membrane structural lipids (26,27). EFAD reduces membrane arachidonic acid (AA) levels, but has no effect on membrane phospholipid content (27,72,73). Reducing membrane AA levels could affect membrane fluidity (55).

Several years ago Ferber and Resch (55) showed that after lymphocyte activation by mitogens or antigens, there was a rapid and preferential incorporation of unsaturated fatty acids (predominantly AA) into membrane phospholipids. This increased the ratio of unsaturated fatty acids to saturated fatty acids (USFA:SFA) in the activated lymphocyte membrane, thereby increasing membrane fluidity (55).

Enhanced membrane fluidity may be important for lymphocyte activation by directly affecting a number of cell processes, including membrane transport. Recently, Pilch et al. (66) showed that unsaturated fatty acids stimulated glucose uptake when added directly to suspensions of adipocyte plasma membranes. Also, in a detailed study of membrane fluidity and glucose transport kinetics, Yuli (67) showed that the maximal rate of glucose transport was closely correlated with increased membrane fluidity up to a peak value, after which further increases in fluidity decreased transport. But membrane fatty acid rearrangements accompanying EFAD may actually increase the ratio of USFA:SFA in membrane phospholipids (27), suggesting that EFAD may alter lymphocyte function in a manner unrelated to membrane fluidity.

Besides affecting membrane structural properties such as fluidity, membrane lipids may also affect membrane protein function. It is becoming increasingly clear that membrane fatty acids are organized into discrete domains within the lipid bilayer (60). Within these domains, specific fatty acids are complexed to membrane proteins (61). This close association between membrane lipids and proteins suggests that alterations in membrane fatty acid composition may affect membrane protein function. This is supported by studies of EFAD and membrane-bound enzymes (26-28) and receptors (62,63). In lymphocytes, EFAD could alter antigen receptor binding, or the binding of membrane transport

proteins to metabolites.

In addition, by lowering membrane AA content, EFAD may reduce the synthesis of prostaglandins and related AA metabolites (54). This could influence lymphocyte activation by directly affecting a number of cell processes, including metabolite transport. In two recent reports Bass, et al (39,40) showed that AA metabolism through the lipoxygenase pathway to form 12-HPETE enhances hexose transport into human polymorphonuclear leucocytes. This suggests a direct role for AA metabolites in transport processes. In a related report, when an inhibitor of AA metabolism, ETYA (5,8,11,14-eicosatetraynoic acid) was added to mast cell cultures, phospholipid turnover and mediator release were blocked (58). This suggests that AA metabolism, phospholipid turnover, and a cellular effector response are linked. It further demonstrates the importance of AA metabolism in cell function. In EFAD, decreased availability of AA could reduce lymphocyte responses in a similar manner.

Although EFAD impairs AIB uptake into Con A-activated lymphocytes, AIB uptake into unstimulated or resting EFAD lymphocytes was similar across the treatments. This suggests a unique role for EFA in activated cells. Since enhanced activity of the high affinity component of neutral amino acid transport accompanies mitogen activation (71), it's possible that EFA may be needed for the full expression of this augmented transport component.

In addition to studying the effects of EFAD on lymphocyte functions, I also looked at the effects of a high PUFA diet on these responses. As in previous reports (12,13) and earlier parts of this thesis, I found that lymphocytes from mice fed the high PUFA diet responded similar to those from control fed mice.

Essential Fatty Acid Deficiency and Lymphocyte Protein Synthesis, RNA Synthesis, Total Protein, and Viability

Introduction

The activation of lymphocytes by mitogens leads to enhanced turnover of membrane components (17), increased metabolite fluxes (17,68-71), and increased synthesis of cellular macromolecules such as protein, RNA, and DNA (17). In previous sections I found that within the first few hours of lymphocyte activation certain aspects of metabolite transport and membrane phospholipid metabolism were reduced in EFAD spleen cells compared with the control or high PUFA groups, provided experiments were performed in lipid free media. After several days in culture, no differences were observed across the treatments in ^{3}H-thymidine incorporation in response to T and B cell mitogens and MLR. The next series of experiments assessed events occurring near the midpoint of lymphocyte activation : protein and RNA synthesis. In addition, total protein and viability were also assessed. All of these experiments were performed in lipid free cultures.

Materials and Methods

^{14}C-Leucine Incorporation into TCA Precipitated Protein. Spleen cells were isolated from mice as described in earlier

experiments and mononuclear cells separated from red cells, dead cells, and granulocytes by centrifugation over Lympholyte M. At the onset of the cultures Con A activated cells received an optimal dose of Con A and 0.15 uCi of ^{14}C-leucine, resting or unstimulated cells received the ^{14}C-leucine alone. All cultures were carried out at a final cell concentration of 5 x 10^6 cells/ml in a final volume of 0.2 ml. Culture medium contained RPMI plus 25 mM HEPES, 1 mM glutamine, 0.2 ml penicillin streptomycin/100 ml culture medium, 5 x 10^{-5} M 2-mercaptoethanol, and .15% dl-FCS. At the specified times cells were removed from incubation and washed twice with 10 volumes of ice cold culture medium. Cell pellets were then solubilized with 1 N NaOH and placed in a 56^{o} shaking water bath for 10 minutes. Two ml of ice cold 10% TCA was then added to the tubes and they were placed in the cold (4^{o}) for 24 hours. Precipitated protein was collected on filter papers using a Millipore sampling manifold, washed twice with cold 10% TCA, dried, and counted. Results are expressed as cpm ^{14}C-leucine in protein/well.

^{3}H-Uridine Incorporation into RNA. Cultures of splenic mononuclear cells were prepared as previously described at a final cell density of 2.5 x 10^6 cells/ml. After 24 hours in culture medium identical to that used in the protein synthesis experiments, 0.1 uCi of ^{3}H-uridine was added and the culture was continued for an additional 18 hours.

At this point cells were collected on filter paper using the sampling manifold, washed twice with 10 ml of PBS, 5 ml of 10% TCA, and 5 ml of methanol. Filter papers were then dried and counted. Results are expressed as cpm ^{3}H-uridine incorporated into RNA/well.

Viability. Viability was determined by trypan blue exclusion throughout all experiments. In most instances 10 ul of 1% trypan blue in PBS was added to cell suspensions in a 1-2 ml volume. After 1-2 minutes a drop of the cell suspension was placed on a hemocytometer and two fields were counted. Cells that stained blue were counted (dead cells) as well as those excluding the dye (live cells). The final calculation was: (mean of two fields counted) x dilution factor x 10^{4} (volume of the hemocytometer field) = number of cells/ml. Total numbers of live and dead cells were counted and the results were expressed as % viable cells.

Results

Although not statistically different, ^{3}H leucine incorporation into TCA precipitated protein of resting and Con A activated spleen cells from EFAD mice exceeded uptake into cells from mice fed control and high PUFA diets at all time points measured (Figure 9). Cellular protein was higher (Figure 10) in the EFAD cells, but viability

Figure 9. ^{14}C leucine incorporation into TCA precipitated protein of splenic mononuclear cells from mice fed the experimental diets. 1 x 10^6 splenic mononuclear cells were incubated with optimal mitogenic doses of Con A (solid lines) or without mitogen (dotted lines). At the times indicated, cells were removed, washed, and precipitated with 10% TCA. Each data point represents the mean ± SEM of duplicate observations from each of 3-5 animals/treatment after a 70-84 day feeding period beginning when the mice were 21±3 days of age.

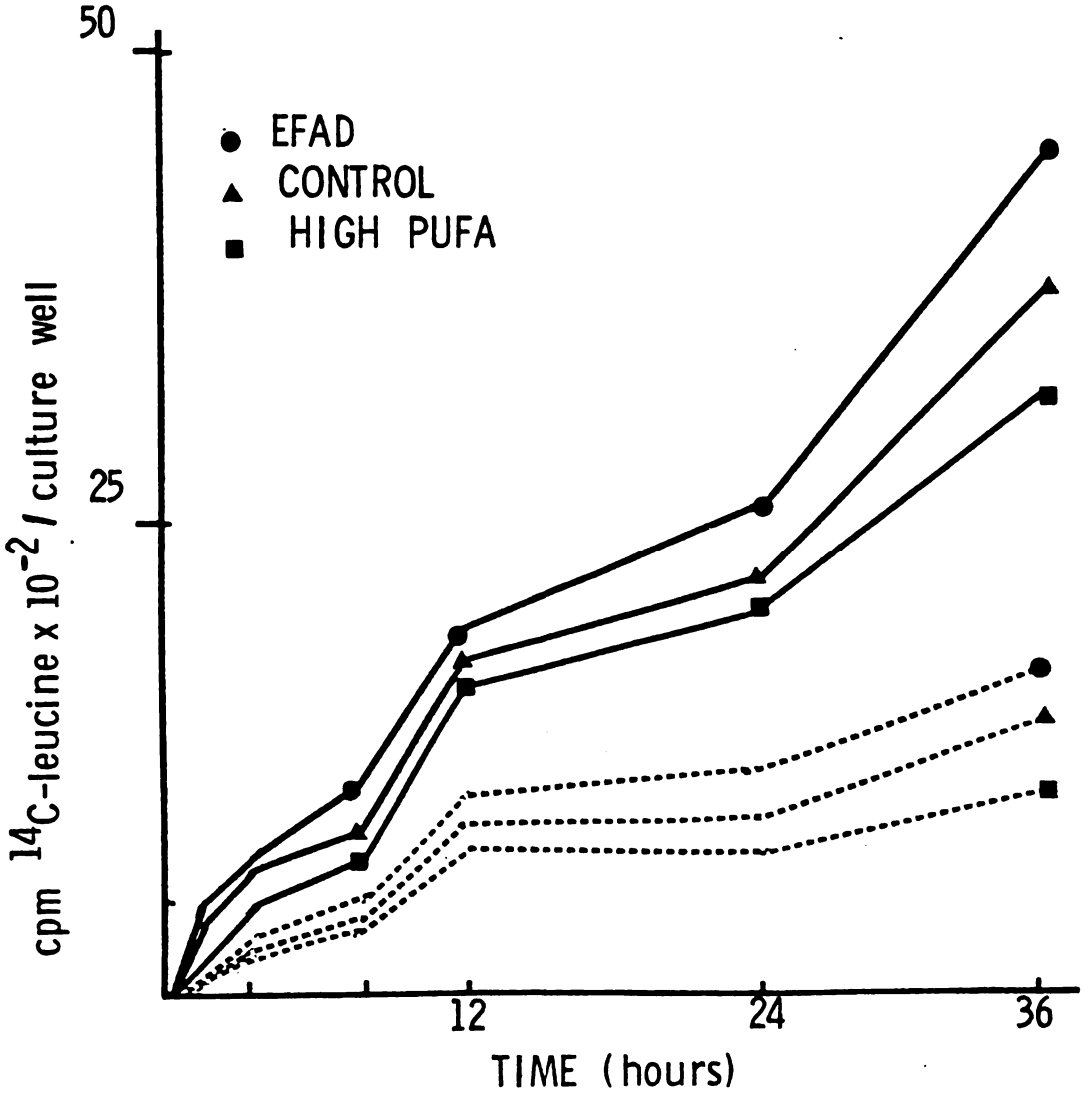
50
25
EFAD
CONTROL
HIGH PUFA
cpm ^{14}C-leucine x 10^{-2} / culture well
12
24
36
TIME (hours)

Figure 9.

Figure 10. Protein content of splenic mononuclear cells from mice fed the experimental diets. 1×10^6 splenic mononuclear cells were incubated for the specified times with optimal mitogenic doses of Con A (solid lines), or without Con A, (dotted lines). Data represent the mean ± SEM of five cultures/dietary treatment.

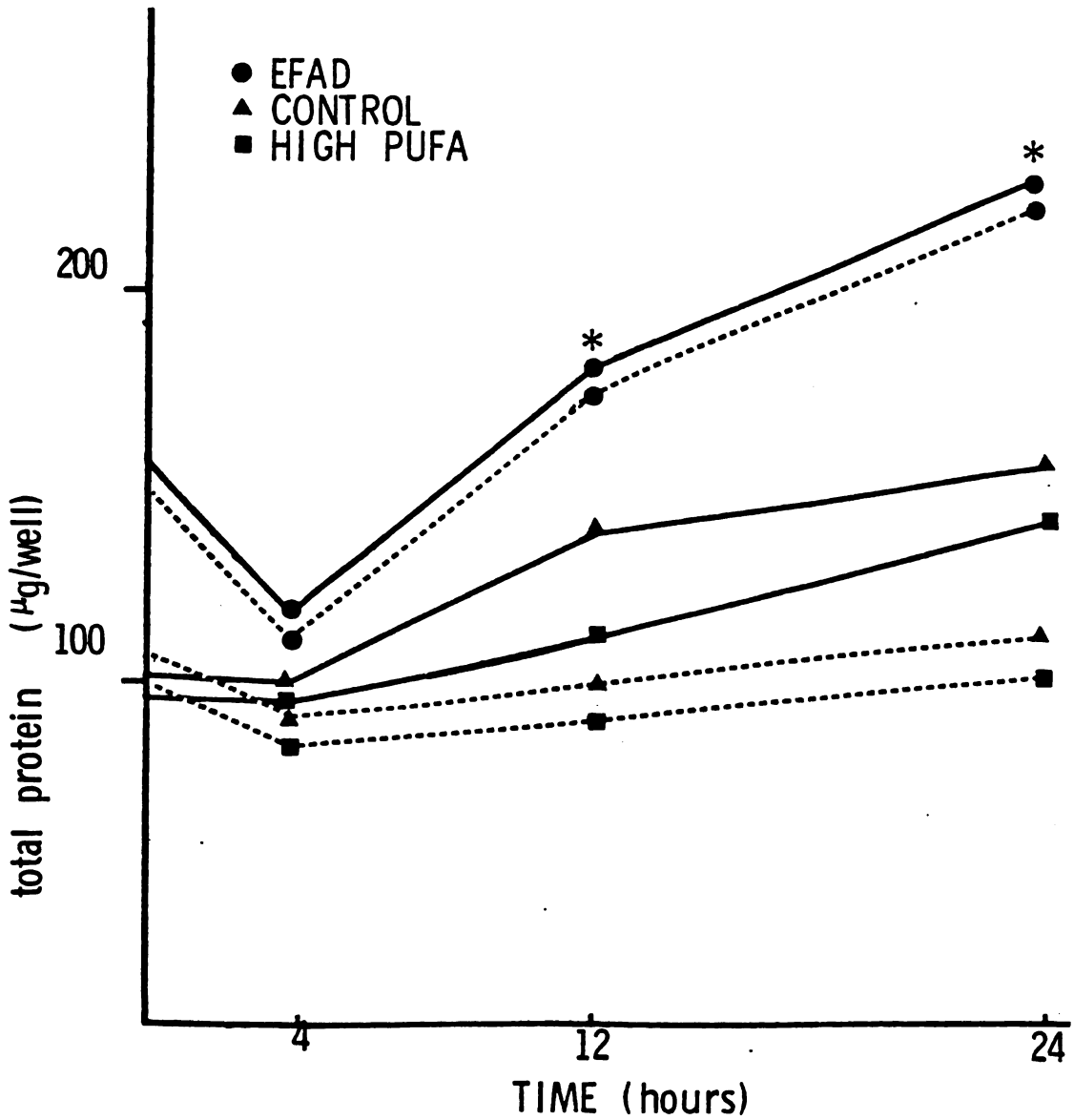
EFAD
CONTROL
HIGH PUFA
200
100
total protein (µg/well)
4
12
24
TIME (hours)
*
*

Figure 10.

did not differ across the treatments (Table 6). This points to a markedly higher protein content in the EFAD cells compared with the other two treatments. RNA synthesis was estimated by measuring the incorporation of ^{3}H-uridine into TCA precipitated RNA. As shown in Table 7, incorporation of ^{3}H-uridine into RNA did not differ across the treatments.

Discussion

During lymphocyte activation by mitogens a number of cellular events occur which have been correlated with blast cell formation (17,69). In this section, protein and RNA synthesis were assessed. Although not statistically different, ^{3}H-leucine incorporation into cellular protein was higher in both unstimulated (resting) and Con A activated EFAD spleen cells. This was unexpected because neutral amino acid (NAA) transport was impaired in EFAD cells. Presumably if decreased NAA transport affects lymphocyte function, it would be detected through assessment of protein synthesis. Although this seems logical it is important to consider that these two experiments measured different aspects of protein metabolism. The NAA transport experiment, which used AIB, indicated that the Km but not the Vmax for NAA uptake was defective. On the other hand, in the present experiment, incorporation of ^{14}C-leucine into TCA precipitated protein was measured. The conditions of the AIB study included measurements of uptake when extracellular

Table 6. Effect of Time in Lipid Free Cultures on % Viability of Spleen Cells From Mice Fed the EFAD Control, or High PUFA Diet.[1]

Time (Hours)	EFAD	Control	High PUFA
4	88	85	83
	±4	±5	±5
8	85	76	71
	±4	±5	±5
16	70	67	64
	±5	±5	±4
24	73	65	64
	±3	±4	±3

[1]Values represent the mean ± SEM of 5 observations per treatment.

Table 7. ^{3}H-Uridine Incorporation Into RNA of Spleen Cells From Mice Fed the EFAD, Control, or High PUFA Diet.[1]

Diet	Unstimulated	Con A
EFAD	865	3291
	±63	±214
Control	790	3800
	±55	±202
High PUFA	579	3767
	±36	±200

[1]Data represent cpm ^{3}H-uridine in TCA-precipitated RNA/culture well. Values are means ± SEM of duplicate observations from 8 mice/treatment.

AIB concentrations were limited. At the lower extracellular AIB levels (0.01 mM), defective transport was observed. The protein synthesis experiments were performed under conditions of NAA excess. Thus, even if the affinity of the transporter was impaired, in NAA excess this defect may not be detected and no effect on protein synthesis would be expected.

What does show, however, is that under conditions of NAA excess, protein synthesis proceeds normally, if not at an elevated level in EFAD spleen cells. This indicates that EFAD does not affect cytoplasmic protein synthesis machinery.

Both resting and Con A activated EFAD spleen cells contain almost double the protein content of the control and high PUFA groups. After 24 hours in culture, the significance of the higher protein content of EFAD spleen cells is unclear, particularly as it relates to lymphocyte activation. It has been demonstrated that EFAD cells have increased Na^+K^+-ATPase units and increased Con A binding sites (28,29), suggesting that some cellular proteins are present in greater amounts in EFAD cells compared with normal cells. In contrast to total protein, ^{3}H-uridine incorporation into RNA and viability were not elevated in the EFAD spleen cells compared with the control and high PUFA groups.

The lack of dietary treatment effects on protein

and RNA synthesis is consistent with previous experiments requiring extended culture times. Of particular interest was the finding that the protein content of the EFAD cells was almost twice that of the other two groups. Although the significance of this is unknown, it points out a form of metabolic adaptation that accompanies EFAD.

Effect of EFAD and Control Mouse Serum on Mitogen Responses

Introduction

As previously discussed EFAD reduces in vivo plaque forming and delayed type hypersensitivity responses, while a high PUFA diet has no adverse effects on these responses (12,13). Attempts to study the relationship between EFAD and immune functions in long term cultures using FCS supplemented media were inconclusive, possibly because of "repair" of the EFAD cells by EFA in the FCS. I then reasoned that one way to prevent in vitro repair was to culture spleen cells from the EFAD mice in media supplemented with serum from EFAD mice. Likewise, spleen cells from control mice were cultured in media supplemented with serum from mice fed the control diet. In addition, cross-over studies were performed in which EFAD spleen cells were cultured in control serum and control spleen cells in EFAD serum. The results of these experiments are presented in this section.

Materials and Methods

Mitogen Experiments. Spleen cell preparations, cell culture, ^{3}H-thymidine labelling, and cell harvesting was performed exactly as described in earlier mitogen experiments (p.47). Dose response curves for each of the mitogens used were obtained, but only the data from the optimal mitogenic doses are presented.

Preparation of Mouse Serum. ICR mice were used as sources of serum for the cultures. These mice were obtained at about 4 weeks of age and fed ad libitum either the EFAD or control diet for 42-56 days before bleeding. Only fresh serum was used in the experiments. In most cases mice were bled the afternoon before an experiment and the blood left to clot overnight in the refrigerator. The serum was collected the next morning and used that day for the experiment.

Results

The data (Table 8) clearly show that the mitogen responses of EFAD and control spleen cells were significantly better in EFAD serum supplemented cultures compared with responses in cultures supplemented with control serum. Con A responses for both treatments were 50% higher in the EFAD serum while PHA and LPS responses were 200-300% higher

Table 8. Effects of EFAD and Control Serum on Mitogen Responses of Spleen Cells From Mice Fed the EFAD or Control Diet.[1]

EFAD Serum	Mice	
	EFAD	Control
Unstimulated	406±75	367±61
Con A	42,852±1164[a]	39,302±1910[a]
PHA	32,855±1657[a]	30,581±1394[a]
LPS	3,917±372[a]	3,837±392[a]
Control Serum		
Unstimulated	378±42	280±32
Con A	28,130±1320	24,093±1181
PHA	11,940±972	14,507±2484
LPS	1,497±218	1,774±266

[1]Values are mean ± SEM of triplicate observations from each of 8 mice/treatment.

[a]Significantly greater than response in control serum.

in the EFAD serum cultures compared with the control serum cultures. Values for the unstimulated cultures did not differ between the two serum supplemented cultures.

Discussion

In light of in vivo results showing that EFAD impairs plaque forming responses and delayed type hypersensitivity it was completely unexpected that EFAD serum would enhance in vitro mitogen responses. But even though these findings do not agree with previous in vivo findings, they are in close agreement with the findings of Kollmorgen et al (85). They found that mitogen responses were greater in cultures with serum from animals fed a low fat diet than mitogen responses in cultures supplemented with serum from mice fed a high PUFA diet. As previously discussed in the literature review, hyperlipoproteinemic serum also inhibits in vitro lymphocyte transformation (87). But neither my control mice (nor high PUFA mice), nor the hyperlipoproteinemic patients are immunodeficient.

Since Con A binds to serum lipoproteins containing Apo B (140), it is possible that Con A responses may be reduced in serum with elevated Apo B. But raising the dose level of Con A in cultures did not overcome the inhibitory effect and there is no evidence to date indicating that PHA or LPS are bound up by serum lipoproteins. Thus, it is unclear how serum, or lipoporteins, from animals fed moderate to

elevated levels of PUFA inhibit lymphocyte responses to mitogens. Presently there are a number of laboratories looking at the immunoinhibitory effects of lipoproteins. It seems that studies should be expanded at this point to include dietary experiments, rather than focusing exclusively on in vitro experimentation. Progress in defining the relationship between dietary PUFA intake, lipoprotein metabolism, and immune functions should provide a basis for interpreting some of the seemingly inconsistent findings reported in these experiments.

CONCLUSIONS

The purpose of this research was to investigate the effects of dietary EFA on various immune functions. The first experiments discussed here were in close agreement with my previous studies (12). Both reports support the conclusion that high PUFA diets do not adversely affect in vivo immune functions, but EFAD impairs in vivo immune functions. That these findings are consistent in plaque forming and delayed type hypersensitivity responses casts some doubt on the proposal that PUFA impair immune responses and "PUFA deficiency" enhances immunity. The findings reported here could be expanded upon by other assays of in vivo immunity. In particular, it would be interesting to look at the effects of the diets used here on allograft rejection, as this system was used to propose an immuno-inhibitory role for PUFA.

The relationship between EFAD and immunity has not been extensively studied. The data presented here should be a useful basis for additional studies. From the standpoint of applied nutrition it seems worthwhile to continue studying the relationship between EFAD and immunity because of the possibility of this deficiency developing in patients receiving total parenteral nutrition.

At the level of basic research further study of EFA and immunocompetent cells would also seem appropriate. The evidence presented here point to a role for EFA in lymphocyte activation. Some effects at the level of the lymphocyte membrane have been demonstrated. Further studies could develop the mechanism of this effect by looking at the effects of EFAD on membrane bound enzymes and receptors. On the other hand, EFAD may affect lymphocyte function by altering the generation of EFA metabolites. This may affect lymphocytes by impairing some intracellular process associated with activation. Alternatively, EFAD may reduce the generation of PG and related lipids by macrophages, and loss of these regulators may affect lymphocyte function.

The effects of EFA metabolites on lymphocyte function is under intense investigation. The findings of this study indicate that EFAD is an additional factor for consideration in this research.

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