Pancreas
Vol. 11, No. 1, pp. 26–37

Essential Fatty Acid Deficiency Prevents Autoimmune Diabetes in Nonobese Diabetic Mice Through a Positive Impact on Antigen-Presenting Cells and Th2 Lymphocytes

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Summary: Protective effects of essential fatty acid deficiency (EFAD) on autoimmunity were shown in rodents. Our goal was to investigate the mechanisms of EFAD effects on autoimmune diabetes in nonobese diabetic (NOD) mice. Weanling female mice were randomized between a control diet group and an EFAD diet group, and the development of diabetes and immune response was determined over a 6-month period. The cumulative incidence of diabetes was significantly reduced in the EFAD group (20 vs 68.75% in the control group; $p < 0.01$), without affecting the insulitis process. Splenocyte reactivity to phytohemagglutinin and anti-CD3 antibody was significantly increased in EFAD-fed mice ($p < 0.01$). The EFAD group also exhibited a dramatic increase in baseline (29-fold) and antigen-presenting cell (APC)-stimulated (10-fold) T cell responses in syngeneic mixed leukocyte reaction. These responses were associated with a marked increase in splenocyte interleukin-4 (IL-4) production, a reduction in interferon-γ production, and a down-regulation of CD45RB isoform expression. Macrophages in the EFAD group exerted a reduced suppressive effect on concanavalin A-induced splenocyte proliferation and were found to release increased amounts of tumor necrosis factor-α and IL-1 and reduced amounts of prostaglandin E_2. These results clearly demonstrate that EFAD prevents diabetes in NOD mice. The data suggest an enhanced activity of Th2-like cells, as well as an effect on APC activity linked to alteration in eicosanoid metabolism. **Key Words:** Autoimmunity—Nonobese diabetic mouse—Eicosanoids—T cells—Lymphokine—Fatty acids.

Essential fatty acid deficiency (EFAD) has been shown to exert protective effects against autoimmune diseases in rodent models. This beneficial influence of EFAD was initially reported for immune-mediated glomerulonephritis of murine lupus (1). Dietary intervention by the elimination of essential fatty acids was also reported to prevent multiple low-dose streptozotocin-induced diabetes in CD-1 mice (2), as well as spontaneous autoimmune diabetes in the BB rat model (3). So far, effects of this diet on diabetes have not been reported with the nonobese diabetic (NOD) mouse, a well-established murine model of autoimmune diabetes. Moreover, the mechanisms of the immunomodulation induced by this diet have to be elucidated. Few studies have examined the biological effects of EFA deficiency on immune responses.

Many studies in the NOD mouse have suggested the presence of immune regulatory cells that can be elicited by various manipulations. Most reports point out that CD4 T cell subsets play a regulatory role in the development of autoimmune diabetes (4–6). A regulatory role for CD8 cells has also been suggested (7). However, the functional characteristics of these regulatory cells have not been described. It has been suggested that T cell subsets that generate interleukin-4 (IL-4) play a role in the regulation of autoimmune diseases. Murine mature

Manuscript received May 10, 1994; manuscript accepted June 24, 1994.

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CD4 cells have been classified into two main phenotypes, called Th1 and Th2, characterized by their cytokine secretory patterns and by their function (8). Th1 cells secrete IL-2, IL-3, interferon-γ (IFN-γ), and lymphotoxin, and Th2 cells secrete IL-3, IL-4, IL-5, IL-6, and IL-10 (9). Th1 cells mediate delayed-type hypersensitivity, whereas Th2 cells help B cells to generate T cell-dependent antibody responses (8). The potential role of Th2-like effector cells was recently emphasized in allotransplantation (10) or immunodeficiency syndromes (11). Data are now accumulating to suggest that an activation of Th1 effector cells and an alteration of Th2 regulatory cells could characterize diabetes-prone NOD mice (12,13). Indeed, the most recent report on the induction of tolerance to glutamic acid decarboxylase injected intrathymically also suggested that prevention of diabetes could result from the induction of $CD4^+$Th2 cells (14). More recent attempts to characterize regulatory cells focused on CD45 isoforms. An autoaggressive potential has been described for $CD45RA^{high}$ and $CD45RB^{high}$ CD4 T cells in NOD mice (15) and rats (16), respectively. The delineation of Th2 cells on the marker CD45 has suggested that $CD45RB^{low}$ cells contain the Th2 subset (17), but this remains controversial (18,19).

Dietary factors are known to play a crucial role in the modulation of the incidence of diabetes in NOD mice (20,21). Using an EFAD diet as a tool, our goal was to understand the role of dietary fatty acids in the development of regulatory T cells in this model.

METHODS

Animals

NOD mice were obtained from Taconic farm (Germantown, NY, U.S.A.), and a colony was developed in our facility by brother-sister mating. The cumulative incidence of diabetes in this colony at 6 months of age reached 67% in female and 33% in male mice. Weanling 3-week-old female littermates were randomly divided into two groups (control diet versus EFAD diet) to control for variation in the incidence of diabetes between litters and for the vertical transmission of virus. Mice were housed two to five per microisolator in a specific pathogen-free vivarium. Unless specified, all mice used in this study were female, and all experiments were carried out at between 90 and 120 days of age in normoglycemic mice. Each in vitro experiment was conducted with a minimum of three mice per group and repeated at least twice. To test for the strain specificity of some of our findings, C57BL/6 mice were also used as control animals, litter matched and randomized between normal diet and EFAD diet.

Diet

Weanling mice were fed either a standard laboratory mouse chow (Purina 5001) or an EFAD diet (Purina 5803C low-essential fatty acid purified diet), both purchased from Purina Test Diets (Richmond, IN, U.S.A.). Purina 5001 contained casein (vitamin-free; 21%), sucrose (15%), dextrin (43.65%), corn oil (5%), lard (5%), nonnutritive fiber (3%), DL-methionine (0.15%), vitamin mixture (2%), choline chloride (0.20%), and mineral mixture (5%). Purina 5803C was obtained by omitting the lipid, eliminating the dextrin, and increasing the sucrose to 68.65%, and contained <10 μg of linoleate/g and no detectable arachidonate. Diet and tap water were provided ad libitum. Mice were fed with the assigned diet for a minimum of 8 weeks before any in vitro experiment was carried out. Mice used to monitor the development of diabetes were fed the assigned diet for the entire follow-up period, i.e., until 30 weeks of age.

Monitoring for diabetes

A subset of female NOD mice ($n = 31$) was used to determine the incidence of overt diabetes until 30 weeks of age. These mice were monitored weekly for glycosuria with Tes-Tape (Eli Lilly & Co., Indianapolis, IN, U.S.A.). In glycosuric mice, overt diabetes was confirmed by blood glucose measurement with a Glucometer II (Miles Inc., Elkhart, IN, U.S.A.) and defined as glucose values above 300 mg/dl.

Pancreas histology

At the end of the follow-up period, at 30 weeks of age, surviving normoglycemic mice in each group were sacrificed for evaluation of the presence of insulitis. The pancreata were fixed in 10% buffered formalin, paraffin embedded, sectioned at 5 μm (five serial sections/pancreas), and stained with eosin-hematoxylin. The intensity of insulitis was assessed according to the topography of lymphocytic infiltration, i.e., periductal or perivascular, periinsular, or intrainsular.

Measurement of plasma fatty acids

Plasma fatty acids were extracted and converted to methyl esters by the method of Lepage and Roy (22). Fatty acid methyl esters were separated and

quantified on an HP5890A II gas-liquid chromatograph (Hewlett Packard, Avondale, PA, U.S.A.) fitted with a Model 7673A automatic split injection system, with a flame ionization detector. The chromatograph was equipped with a 30-m fused silica capillary column coated with SP-2380 with a 0.32-mm internal diameter and a 0.20-μm film thickness (Supelco, Bellefonte, PA, U.S.A.). Helium flow rate was 1 ml/min, initial pressure setpoint was 5 psi, injector port and detector temperatures were 240°C, and split ratio was 50:1. Quantification was based on the internal standard, tridecanoic acid, which was added to the samples prior to processing. Calibration used a standard mixture of fatty acids (NuCheck Prep, Elysian, MN, U.S.A.).

Mitogen responses

Spleens from age-matched NOD mice of each group were removed aseptically, minced, and depleted of red blood cells by ammonium chloride exposure. The spleen cells were cultured in U-bottom 96-well plates (No. 25850; Corning, NY, U.S.A.) at a density of 5×10^5 cells per well in 200 μl of medium consisting of RPMI 1640 (Sigma, St. Louis, MO, U.S.A.) supplemented with 10% fetal bovine serum, sodium pyruvate (1 m*M*), nonessential amino acids (0.1 m*M*), HEPES buffer (25 m*M*), sodium bicarbonate (24 m*M*), and antibiotics. Cultures were performed in quadruplicate and maintained for 72 h at 37°C in 5% CO_2 in air.

Splenocyte proliferation was induced with concanavalin A (Con A; 4 μg/ml) and phytohemagglutinin (PHA; 5 μg/ml), both purchased from Sigma, or with anti-CD3 monoclonal antibody (mAb) (2.5 μg/ml) purchased from PharMingen (San Diego, CA, U.S.A.). For each mitogen, the entire stimulatory range was tested with both groups of mice to establish the optimal concentration, which was found to be identical in both groups. Synthesis of DNA was estimated from the incorporation of ^{3}H-thymidine (1 μCi/well; Du Pont NEN, Boston, MA, U.S.A.) added during the last 18 h of 72-h cultures.

Syngeneic mixed-leukocyte reaction (SMLR)

SMLR was performed using nylon wool-adherent cells as stimulator cells and nylon wool-nonadherent cells as responder cells, as described previously (23). Briefly, 5 to 10×10^7 splenocytes in 4 ml of RPMI/fetal bovine serum (FBS) medium were run through a column containing 0.6 g of nylon wool (Polysciences, Warrington, PA, U.S.A.) and incubated for 45 min at 37°C in 5% CO_2 in air. Nonadherent cells were eluted with 25 ml of warmed RPMI/FBS. The purity of this T cell preparation was confirmed by labeling cells with fluorescein isothiocyanate (FITC)-Thy 1.2 (Becton-Dickinson, Mountain View, CA, U.S.A.), and yields of >85% purity were consistently obtained. Adherent cells (macrophages, dendritic cells, and B cells) were obtained by tearing the nylon wool with forceps in a petri dish. Adherent cells were collected and treated with 50 μg/ml of mitomycin C (Sigma) for 40 min at 37°C in 5% CO_2 in air, washed three times in RPMI, and used as stimulators. Responder cells (5×10^5) and stimulator cells (5×10^5) were cultured in 96-well plates for 5 days at 37°C in 5% CO_2 in air and pulsed with 1 μCi/well of ^{3}H-thymidine during the final 18 h.

Measurement of lymphokine production from splenocytes

Splenocytes were plated in U-bottom 96-well plates at a density of 5×10^5 cells per well in 200 μl of RPMI medium. Cells were stimulated by soluble anti-CD3 mAb (2.5 μg/ml) as described previously (24). Previous kinetic studies have shown that IL-4 mRNA reached a peak on day 2 following stimulation with anti-CD3 mAb of murine splenocytes (25). Thus IL-4 and IFN-γ were assayed in 36-h culture supernatant by two-site enzyme-linked immunosorbent assay (ELISA) using purified anticytokine capture mAb and biotinylated anticytokine detecting mAb, with reference to standard curves constructed using known amounts of recombinant lymphokine. Both mAb pairs and recombinant lymphokines were obtained from PharMingen.

Flow cytometric analysis

Two-color flow cytometric analysis was performed using an FACScan 440 (Becton Dickinson, Mountain View, CA, U.S.A.) linked to a Hewlett-Packard computer (15). Splenocytes (1×10^6 cells), after hemolysis of red blood cells with 0.015 *M* Tris–0.83% NH_4Cl, were incubated on ice for 30 min with the previously titrated appropriate antibody, then washed three times in the staining buffer, consisting of phosphate-buffered saline (PBS) supplemented with 1% heat-decomplemented FBS and 0.1% sodium azide. Immunofluorescence staining was performed with FITC-conjugated 16A (rat IgG2a directed against mouse CD45RB) and with phycoerythrin-conjugated RM4-5 (rat IgG2a anti-mouse CD4). Controls consisted of FITC-

conjugated rat IgG2a κ Ig isotype standard. Each antibody was purchased from PharMingen. Cell size was estimated by forward light scatter, and gating was based on forward- and side-angle light scatter. Five thousand cells were analyzed from gated preparations.

Macrophage cultures and lymphokine production

Peritoneal macrophages were obtained by sterile lavage using α-minimum essential medium (α-MEM) supplemented with 2% FBS, sodium bicarbonate (24 m*M*), L-glutamine (2 m*M*), and antibiotics and plated in 96-well plates at a density of 1×10^5 cells per well in 200 μl of medium. Nonadherent cells were removed after 3 h at 37°C in 5% CO_2 in air. Macrophages were stimulated with lipopolysaccharides (LPS; from *Escherichia coli* 055:B5; 10 μg/ml) purchased from Sigma, and 24-h culture supernatants were assayed for tumor necrosis factor-α (TNF-α) and IL-1 by two-site ELISA using kits supplied by Genzyme (Cambridge, MA, U.S.A.).

Macrophage effect on Con A-induced splenocyte proliferation

To assess the regulatory influence of peritoneal macrophages on mitogen-induced splenocyte proliferation, splenocytes were cultured at a density of 2.5×10^5 cells per well in 200 μl of medium supplemented with the optimal concentration of Con A (4 μg/ml), in the presence of various concentrations of peritoneal macrophages, as described previously (25). Also, using the same method, macrophages from the EFAD group were cocultured with splenocytes from the control group, and vice versa.

Prostaglandin determination

The prostaglandin E_2 (PGE2) content in supernatants of splenocyte or macrophage 24-h cultures was determined by an enzymeimmunoassay system (Amersham, Arlington Heights, IL, U.S.A.). This solid-phase assay used a peroxydase-labeled PGE2 and an anti-PGE2 antibody, with a cross-reactivity of 7% with PGE1 and 4.3% with PGF2α and a sensitivity of 16 pg/ml.

RESULTS

Validation of EFA deficiency (Table 1)

All NOD mice in the EFAD group exhibited the usual symptoms of EFA deficiency, such as dermatitis (mild hair loss, periorificial rash, scaly paws) and hepatic fatty changes at autopsy, present to a minor degree after 8 weeks and worsening during the follow-up period. However, after 8 weeks of experimental diet, no significant change in total body weight was observed (23.16 ± 0.65 g in group C vs 23.89 ± 0.57 g in group EFAD; p = ns) comparing nondiabetic NOD mice in each group. During the follow-up period, no significant growth retarding effect was evidenced (at 21 weeks of diet, 25.80 ± 0.34 in group C vs 24.28 ± 0.97 g in group EFAD; p = ns). In contrast, C57BL/6 mice were dramatically more susceptible to the characteristic changes of EFA deficiency and experienced an early and severe weight loss (data not shown). Plasma fatty acid profiles, shown in Table 1, confirmed that NOD mice in the EFAD group exhibited a marked depletion in (n-6) fatty acids linoleate and arachidonate, as well as an increase in (n-9) oleate and (n-7) palmitoleate. The 16:1 (n-7)/18:2 (n-6) ratio was significantly increased in EFAD group, thus meeting the criteria for essential fatty acid deficiency (26).

Incidence of diabetes in female NOD mice fed with EFAD diet (Fig. 1)

As shown in Fig. 1, control mice developed diabetes beginning at 12 weeks of age, and the cumulative incidence of diabetes reached 68.75% by 30 weeks of age. In contrast, the onset of overt diabetes was delayed to 16 weeks of age, and the incidence at 30 weeks of age was significantly reduced in the EFAD group, with a cumulative incidence of 20% at 30 weeks of age ($p < 0.01$, log-rank test). However, histological examination of the pancreas taken from nondiabetic NOD mice at 30 weeks of age revealed similar degrees of insulitis in the EFAD group and in the control group (Fig. 2).

TABLE 1. *Fatty acid analysis of plasma from female NOD mice on control and EFAD diets*

	mol %				
NOD group	16:1 (n-7) palmitoleate	18:1 (n-9) oleate	18:2 (n-6) linoleate	20:4 (n-6) arachidonate	16:1 (n-7)/18:2 (n-6) ratio
Control (n = 5)	1.63 ± 0.86	20.30 ± 2.55	21.16 ± 2.11	7.31 ± 0.77	0.10 ± 0.07
EFAD (n = 5)	5.90 ± 0.57*	37.73 ± 1.19*	4.35 ± 0.45*	3.44 ± 0.75*	1.47 ± 0.29*

Results are expressed as mol% of total fatty acids present. Data are mean values ± SEM. *$p < 0.005$, EFAD vs control.

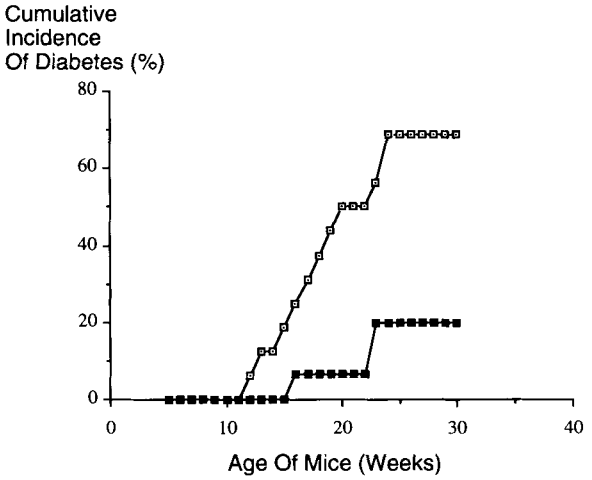

FIG. 1. The EFAD diet prevented the onset of spontaneous diabetes in the NOD mouse. Female NOD mice, litter matched, were randomized at weaning between a normal mouse chow and an essential fatty acid-deficient diet and checked weekly for the occurrence of diabetes. The occurrence of diabetes was delayed and the cumulative incidence significantly reduced in the experimental group (■) (n = 3/15; 20%) compared to the control group (⊡) (n = 11/16; 68.75%; $p < 0.01$, log-rank test).

Effect of EFAD diet on the proliferative responses of splenocytes (Figs. 3 and 4)

Proliferative responses of splenocytes were examined in vitro through stimulation with various mitogens and with syngeneic antigen-presenting cells (APC) in the SMLR. The optimal dose of each mitogen was titrated in dose-response experiments and was found to be identical in both groups (data not shown). The responses to PHA and anti-CD3 mAb were significantly increased in mice fed the EFAD diet, by 167 and 216% of control levels, respectively, whereas the response to Con A was significantly decreased, to 82% of controls ($p < 0.01$; Fig. 3). Interestingly, baseline splenocyte proliferation, without any mitogen stimulation, was dramatically increased by 372% in the EFAD group (Fig. 3).

We also studied the SMLR, which is a model of in vitro T cell proliferation in response to syngeneic MHC class II antigens, since NOD mice are known to exhibit low responsiveness in this assay (27). As shown in Fig. 4, female control NOD mice showed

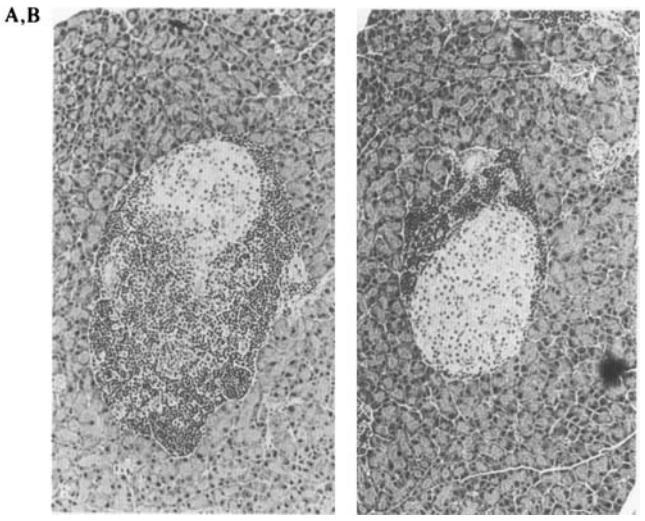

FIG. 2. The EFAD diet did not prevent the occurrence of spontaneous insulitis in the NOD mouse. Histological examination of the pancreas taken from nondiabetic NOD mice at 30 weeks of age revealed similar degrees of insulitis in the control group (A) and in the EFAD group (B).

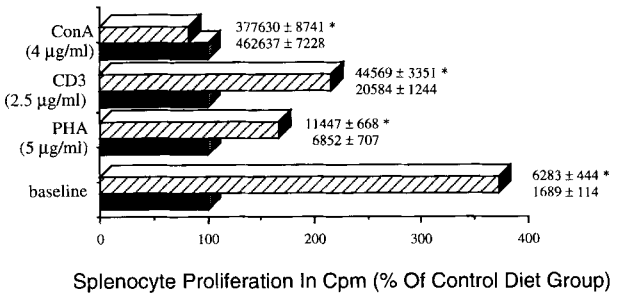

FIG. 3. EFAD changes mitogenic responses in splenocytes of female NOD mice. Splenocytes (5×10^5) were cultured for 72 h in 200 µl of medium supplemented with a mitogen and were pulsed during the last 18 h with ^{3}H-thymidine. Absolute counts per minute (cpm) values are mentioned for each group. Cultures were performed in quadruplicate. Data are shown as means ± SEM (three mice/group). Responses are normalized to facilitate comparisons between the two groups (control diet values = 100%). *$p < 0.01$, EFAD (▨) vs control (■).

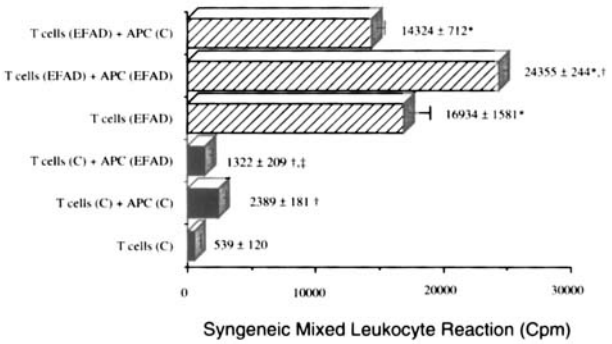

FIG. 4. The EFAD diet enhances T cell responses in SMLR in female NOD mice. Responder T cells (5×10^5) from the control group (C; ■) or the EFAD group (EFAD; ▨) were cultured in quadruplicate for 5 days in 200 µl of medium and stimulated with syngeneic APC. APC were harvested either from the same group as T cells or from the opposite group and treated with mitomycin (thymidine uptake: 67 ± 45 cpm in group C and 132 ± 70 cpm in the EFAD group). Data are shown as means ± SEM. *$p < 0.001$, EFAD vs control; †$p < 0.001$, T + APC vs T alone; ‡$p < 0.01$, T + APC (C) vs T + APC (EFAD).

a T cell baseline response of 539 ± 120 cpm that was weakly stimulated (2,389 ± 181 cpm) in the presence of syngeneic APC. In contrast, female EFAD NOD mice exhibited a dramatic enhancement of baseline T cell response, to 16,934 ± 1,581 cpm, which was further increased to 24,355 ± 244 in the presence of APC. We also cocultured T cells from control diet-fed mice with APC from EFAD diet-fed mice, and vice versa. Interestingly, T cells from the EFAD group were not further stimulated by APC from the control group and exhibited a response similar to the baseline response (14,324 ± 712 vs 16,934 ± 1,581; p = ns). Conversely, T cells from the control group were no more responsive to APC from the EFAD group than to APC from the control group (1,322 ± 209 vs 2,389 ± 181; p = ns; Fig. 4).

Evaluation of splenocyte subsets by cytokine secretory patterns (Table 2) and flow cytometry analysis (Table 3)

We tested the hypothesis that an EFAD diet could have modified the functional differentiation and subset distribution of splenocytes. These analyses were performed on 24-week-old female NOD mice. The Th1 cytokine IFN-γ and Th2 cytokine IL-4 were measured in 36-h culture supernatant of anti-CD3 mAb-stimulated splenocytes. As summarized in Table 2, NOD mice in the EFAD group exhibited a dramatic increase in IL-4 secretion levels, concomitant with a reduction in IFN-γ levels, suggestive of an increase in Th2 activity and a decrease in Th1 activity.

Expression of CD45RB isoform also significantly differed among the two populations. As shown in Table 3, a significant decrease was observed in the EFAD group in the total expressions of both CD4 (26.03 ± 0.90 vs 34.01 ± 1.86%) and CD45RB (40.52 ± 0.59 vs 62.15 ± 0.22%). Among the CD45 RB^+ splenocytes, a high-intensity subset was observed ($CD45^{+bright}$) containing only a small percentage of $CD4^+$ cells (<5%). This $CD45^{+bright}$ subset also decreased in the EFAD group (9.80 ± 1.88 vs 17.74 ± 1.38%).

TABLE 2. *EFAD diet enhances IL-4 production and reduces IFN-γ production from anti-CD3 mAb-treated splenocytes in female NOD mice*

NOD group	IL-4 (pg/ml)	IFN-γ (U/ml)
Control	8.7 ± 0.3	1,438 ± 283
EFAD	4,683 ± 1,120	486 ± 246

IL-4 and IFN-γ levels were determined by an ELISA method using recombinant cytokines to generate a standard curve and pairs of purified mAb (PharMingen) in 36-h supernatants from splenocytes stimulated with anti-CD3 mAb. Values are means ± SEM. n = 3 mice in each group; one of three experiments is shown.

TABLE 3. *EFAD diet affects the distribution of splenocyte subsets in female NOD mice*

NOD group	$CD4^+$ (%)	CD45 RB^+ (%)	$CD45^{+bright}$ $CD4^-$ (%)
Control (n = 5)	34.01 ± 1.86	62.15 ± 0.22	17.74 ± 1.38
EFAD (n = 5)	26.03 ± 0.90*	40.52 ± 0.59*	9.80 ± 1.88*

Fluorescence-activated cell sorting analyses were performed as described under Methods. n = number of mice per group. Results are means ± SEM. *p < 0.01.

Effect of EFAD on PGE2 release from splenocytes (Table 4)

PGE2 release was measured in 24-h culture supernatant of splenocytes stimulated with Con A (4 μg/ml). As shown in Table 4, PGE2 release in the EFAD group was decreased from 1,978 to 1,193 pg/ml (40% decrease; p < 0.001) in female NOD mice.

Macrophage effect on Con A-induced splenocyte proliferation (Fig. 5, Table 5)

Peritoneal macrophages added in excess suppressed Con A-induced splenocyte proliferation in both groups, and this effect was proportional to the concentration of macrophages (Fig. 5). Importantly, at all concentrations tested, the suppressive effect of macrophages was significantly reduced in the EFAD group, compared to the control group (Fig. 5). Moreover, macrophages from the EFAD group cocultured with splenocytes from the control group had a reduced suppressive effect; conversely, splenocytes from the EFAD group exhibited an enhanced suppression when cocultured with macrophages from the control group (Table 5).

TABLE 4. *EFAD diet suppresses PGE2 production in splenocytes of female NOD mice*

NOD group	PGE2 (pg/ml)
Control (n = 5)	1,978 ± 125
EFAD (n = 5)	1,193 ± 87*

The effect of an EFAD diet on immunoreactive PGE2 production was measured in 24-h culture supernatants of splenocytes (5 × 10^6/ml) stimulated with ConA (4 μg/ml) by an ELISA system. All experiments were performed in quadruplicate wells. n = number of animals in each group. Values are means ± SEM. *p < 0.001, EFAD vs control.

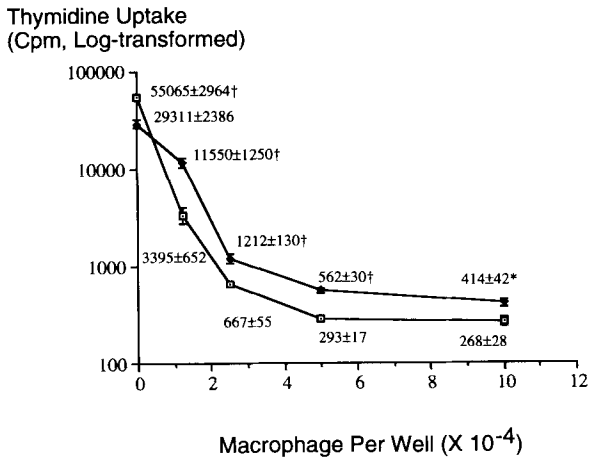

FIG. 5. The EFAD diet reduces the suppressive effect of peritoneal macrophages on the splenocyte response to Con A. Proliferative responses of splenocytes (2.5×10^5 per well) cocultured with peritoneal macrophages in the presence of Con A (4 μg/ml) were determined in 72-h cultures. Macrophages in excess exert a suppressive effect, in relation to their concentration. This effect is significantly reduced in the EFAD group at all concentrations. Data are means ± SEM (three mice/group). *$p < 0.05$ and †$p < 0.005$, EFAD (♦) vs control (▣).

Peritoneal macrophage secretory pattern (Fig. 6)

TNF-α, IL-1, and PGE2 contents were measured in 24-h culture supernatants of macrophages stimulated with LPS (10 μg/ml). As illustrated in Fig. 6, in the EFAD group, compared to the control group, TNF-α release was increased from 318 to 422 pg/ml (33% increase; $p < 0.001$), IL-1 release was increased from 41 to 60 pg/ml (46% increase; $p < 0.01$), and PGE2 release was decreased from 392 to 124 pg/ml (68% decrease; $p < 0.001$).

DISCUSSION

In this study, we demonstrate for the first time that essential fatty acid deficiency induced by an EFAD diet markedly reduces the incidence of diabetes in NOD mice. We also demonstrate specific effects of the EFAD diet on T cell proliferative responses, cytokine production, and T cell populations. In addition, we show an effect of EFAD diet on macrophage prostaglandin and cytokine production. This study provides important information about the effects of EFAD diets on the immune response and suggests potential mechanisms through which the NOD mouse may be protected from diabetes by this dietary modification.

Studies in the NOD mouse, a model for Type 1 diabetes, have shown that dietary manipulation can have profound effects on the incidence of diabetes

TABLE 5. *Peritoneal macrophages from EFAD-fed NOD mice exert a reduced suppressive effect on Con A-stimulated splenocytes in both control and EFAD groups*

Macrophages		Diet	
Control	EFAD	Control	EFAD
0	0	55,065 ± 2,964	29,311 ± 2,386*
+	0	667 ± 55	1,212 ± 130*
0	+	2,863 ± 352	774 ± 148*

Peritoneal macrophages in excess (2.5×10^4 per well) exert a suppressive effect on Con A-induced splenocyte proliferation in both groups. Macrophages from the EFAD group were less suppressive on splenocytes from the control group, and conversely, macrophages from the control group were more suppressive on splenocytes from the EFAD group. Data are means ± SEM (three mice/group). *$p < 0.005$, EFAD diet vs control diet.

(20,21). Interestingly, one of these studies suggested that the presence of an unknown lipoidal substance in some mouse chow exerts a diabetogenic effect (21). Dietary lipids are known to modulate the immune response (28). Furthermore, specific dietary modifications, i.e., EFAD diets, have been shown to suppress markedly the development of several autoimmune disease processes (1–3). Studies in the BB/W rat, a model of autoimmune Type 1 diabetes, documented a similar preventive effect of EFAD diets in these animals that correlated with the depletion of (n-6) fatty acids (3). In this study, we similarly demonstrate that EFA deficiency delays diabetes onset and markedly reduces the incidence of diabetes in NOD mice (see Fig. 1).

Despite the marked reduction in the incidence of diabetes in NOD mice fed EFAD diets, insulitis was

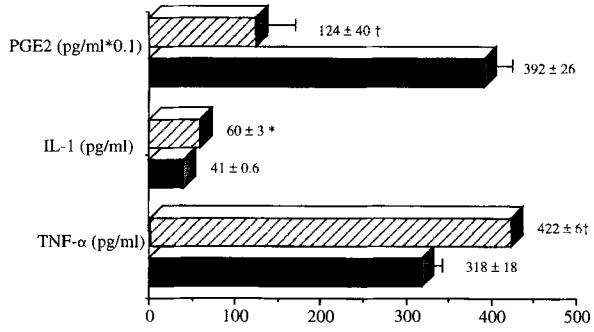

FIG. 6. The EFAD diet influences the production of immunoreactive TNF-α, IL-1, and PGE2 by resident peritoneal macrophages from nondiabetic female NOD mice. Macrophages (1×10^6/ml) were stimulated with LPS (10 μg/ml), and 24-h culture supernatants were assayed for TNF-α, IL-1, and PGE2 by an ELISA method, as detailed in the text. All experiments were performed in quadruplicate wells and included a minimum of three mice per dietary group. Values are shown as means ± SEM. *$p < 0.01$ and †$p < 0.001$, EFAD (▨) vs control (■).

present in EFAD-treated nondiabetic NOD mice. If moderate to severe levels of fatty acid deficiency are required to modulate the immune response, then it is not surprising that insulitis is present in EFAD-treated mice, as 3-week-old weanling mice would not become deficient until 11 weeks of age, a full 6–7 weeks after insulitis has begun (29). The presence of insulitis, and the lack of progression to diabetes, suggests that the EFAD diet modifies this process, perhaps by limiting the effector function of macrophages or T cells that are thought to mediate the destruction of β cells (30,31). Alternatively, EFAD diets may enhance the activity of regulatory networks in the NOD mouse (4–7).

Given that EFAD diets effectively inhibit the development of diabetes, it is important to understand the specific effects of this diet on NOD immune responses. Previous studies examining the effects of EFAD diets on the immune response have suggested that there is an enhanced skin allograft rejection and tumor surveillance (32). A few studies have demonstrated effects on macrophages and have shown that there are reduced numbers of these cells, their major histocompatibility complex (MHC) class II antigen expression is increased, and chemoattractant leukotriene B4 production is reduced, along with a general inhibition of eicosanoid production (33,34). The effect of fatty acid deficiency on the function of T cells, which play a central role in autoimmune diseases, however, has undergone only limited analysis. Finally, it has also been suggested that EFAD-induced alterations in the immune response may be mediated through the effects of fatty acids on membrane fluidity and flexibility, which, in turn, alter the behavior of enzymes and receptors important in cell activation (35).

In this study, we examined the effects of the EFAD diet on several parameters of the NOD immune response. We first analyzed the effects of an EFAD diet on the mitogen activation of T cells. Previously, NOD thymic and splenic T cells have been shown to be hyporesponsive to anti-CD3 mAb (36). The hypoproliferative response of NOD T cells has been thought to be due to defects along the protein kinase C/p21ras/p42mapk T cell activation pathway (37). In addition to T cell proliferation, a recent study has demonstrated that there is limited production of IL-2 and IL-4 when NOD T cells are activated with soluble anti-CD3 mAb (13).

We found that the EFAD diet enhances the response of splenic T cells to soluble anti-CD3 mAb (116% increase) and PHA (67% increase) (see Fig. 3). The reasons for the increased responsiveness of T cells to these mitogens in EFAD-treated NOD mice may be multifactorial. A potential explanation could relate to the increased production of IL-4 (see Table 2). This suggestion is in agreement with a study that demonstrates that IL-4 in vitro reverses NOD T cell unresponsiveness to mitogens (13). The increased proliferative response and IL-4 production from EFAD T cells could result from changes in the T cell membrane that positively affect the function of the T cell receptor and other cell surface ligands. In addition, changes in the T cell populations of NOD mice fed the EFAD diet could also affect IL-4 production and subsequent mitogen responsiveness. In NOD mice fed an EFAD diet, there was a marked increase in the percentage of $CD45RB^-$ cells (see Table 3). Previous studies have demonstrated that $CD45RB^-$ T cells activated by PHA produce predominantly IL-4 (17). Therefore, an increase in this cell population and the concomitant increase in the production of IL-4 following mitogen stimulation may account for the increase in the proliferative activity of T cells from EFAD-treated mice. Furthermore, the $CD45RB^-$ $CD4^+$ T cells, which are thought to be memory cells and are increased by the EFAD diet, respond more readily to activation signals (38). Finally, the enhanced production of IL-1 by macrophages (see Fig. 6), a cofactor for Th2 T cell activation, may also serve to enhance the production of IL-4 (39). Therefore, although we have not defined the mechanism for enhanced responsiveness to PHA and anti-CD3, it may be related to factors that influence IL-4 production.

In comparison to anti-CD3 and PHA, the proliferative response to Con A was not enhanced in T cells from EFAD-treated NOD mice (see Fig. 3). It has previously been shown that NOD splenocyte proliferative responses to Con A were not impaired and were equivalent to those of BALB/c mice (36). The lack of an effect of the EFAD diet on Con A activation of NOD T cells suggests that essential fatty acid deficiency in NOD mice specifically affects T cell activation pathways for anti-CD3 and PHA. It is noteworthy that Th1 and Th2 cells use different transmission pathways after T cell receptor-mediated stimulation, which remain unclarified in the case of Th2 cells (40). We also noted that the response to Con A is suppressed in EFAD-fed NOD mice in comparison to mice receiving control diets. A potential explanation may relate to the 20% reduction in the $CD45RB^+$ population. Studies in rats

have demonstrated that the proliferative response to Con A is greatest in the $CD45R^+$ population at 72 h of culture (41). Therefore, the Con A-induced expansion of splenic T cells in EFAD-fed mice may be reduced due to decreases in their number of $CD45RB^+$ T cells.

The alteration of T cell responses to mitogens induced by the EFAD diet prompted us to investigate further T cell activation in EFAD-treated NOD mice. The activation of T cells by syngeneic or autologous APC appears to be important in the induction of suppressor/inducer $CD4^+$ T cells, which may play a role in the generation of regulatory networks (42). Previous reports have demonstrated that the activation of T cells by syngeneic APC (SMLR) is deficient in NOD and other strains of autoimmune mice, as well as in humans with autoimmune disease (42–44). Defects in cytokine production and in APC appear to contribute to the deficient SMLR in NOD mice (27). The importance of the SMLR is highlighted further by the finding that the incidence of diabetes in various substrains of NOD mice correlates inversely with the responsiveness of the SMLR (43), and pharmacological interventions that increase this response, i.e., lymphotoxin, reduce the incidence of diabetes (23).

We therefore examined the SMLR in EFAD-treated NOD mice. Because a deficient SMLR could arise from defects in APC populations and in T cells, we performed experiments to delineate the effects of the EFAD diet on both populations. We found that the SMLR of EFAD-treated mice was markedly increased, due mostly to the dramatic effect of the EFAD diet on the T cell population (see Fig. 4). We cannot readily explain the large increase in the spontaneous proliferation of T cells from EFAD-treated mice, but it does not appear to be due to contaminating APC, as >90% of the nylon wool-purified cells were Thy 1.2 positive (data not shown). The addition of APC from EFAD-treated mice further increased the proliferative response of EFAD T cells, whereas those from control animals tended to suppress EFAD T cell responses. The APC population from EFAD-treated mice was, however, unable to enhance the SMLR when combined with T cells from control mice. These data demonstrate a major effect of the EFAD diet on T cells, with some additional positive effects on the function of APC. The increased T cell response to syngeneic APC in EFAD mice could result in the more efficient production of regulatory elements in the periphery and thus limit the expression of diabetes in NOD mice.

This major effect of EFAD on T cell reactivity was further investigated by T cell subset analysis. Recent data from the NOD mouse and other autoimmune models suggest that Th2 cells play a role in the regulation of autoimmune diseases (12,13,15, 16,36). We therefore examined the effects of EFAD diet on the production of the Th1/Th2 prototypical cytokines IFN-γ and IL-4 following stimulation with soluble anti-CD3 and APC. This approach to T cell activation has been shown to stimulate both naive and memory cell populations as well as the production of IL-2 and IL-4 (45). As shown in Table 2, the production of IL-4 was markedly increased in EFAD-treated mice, whereas the production of IFN-γ was significantly reduced, suggesting a substantial increase in the activity of Th2 cells. The accompanying reduction in IFN-γ production could be secondary to the effects of IL-4 on its secretion or could reflect an overall reduction in the activity of the Th1 population in EFAD-treated mice. Although we have not determined the mechanism for the increased activity of the Th2 population, it may be related to increased T cell responsiveness and the increased production of IL-1, a cofactor for Th2 activation (40), by macrophages. In addition, it may also be hypothesized from our data that the EFAD diet increases the Th2 cell population, as suggested by the increase in $CD45RB^-$ $CD4^+$ T cells. Although still controversial, some studies have suggested that Th2 cells are $CD45RB^-$, whereas $CD45RB^+$ cells belong to the Th1 subset (17–19). If Th2 cells indeed play a regulatory role in the NOD mouse, the increased activity of this cell population in animals fed an EFAD diet could contribute to the reduction in the incidence of diabetes. Although the available data have suggested that regulatory cells belong mostly to the CD4 subset (4–6), it should be noted that there is a potential for CD8 regulatory cells, characterized by analogous cytokine patterns and CD45 isoforms as CD4 cells (46,47). Overall, our observations of an enhanced T cell reactivity, an increased IL-4 production, a reduced IFN-γ secretion, and an increase in $CD45RB^-$ cells in EFAD-treated mice are suggestive of an induction of Th2-like cells.

In addition to affecting T cells, the EFAD diet enhanced the ability of the APC population to stimulate T cell responses. In experiments involving the SMLR, we found that the APC population from EFAD-treated mice stimulated T cells, whereas

those from control mice suppressed this response. Members of our group have demonstrated previously that macrophages, a component of the splenic APC population, suppress the SMLR (48; manuscript in preparation) and responses to antigens (Clare-Salzler, unpublished data). They and others have found that macrophage suppression is mediated partially by prostaglandins (48,49). In addition, other studies have demonstrated an enhanced metabolism of arachidonic acid in NOD macrophages (50). We therefore examined the effects of the EFAD diet on the macrophage suppression of Con A-activated splenocyte responses and found that macrophages from EFAD-treated mice were significantly less suppressive than those from control NOD mice (see Fig. 5 and Table 5). Because the EFAD diet has been shown to reduce eicosanoid production, and prostaglandins contribute to macrophage-mediated suppression, we evaluated the production of PGE2 by activated macrophages. As shown in Fig. 6, the EFAD diet markedly reduced the production of PGE2. This is consistent with previous studies that have demonstrated similar effects on eicosanoid metabolism in peritoneal macrophages (34). The reduced production of prostaglandins by NOD macrophages from EFAD-treated mice appears to be secondary to the effects of essential fatty acid deficiency on arachidonic acid metabolism (34). The reduction in prostaglandin production would affect the immune responses of NOD mice in several ways. First, prostaglandins inhibit the expression of MHC class II molecules on macrophages and would limit antigen presentation and T cell activation (51). In addition, prostaglandins, through cyclic AMP-mediated mechanisms, are potent inhibitors of T cell proliferation and affect particularly the production of IL-2 (52). Furthermore, the production of prostacyclin has been shown strongly to inhibit IL-4 production (53). Therefore, a reduction in the suppressive effects of macrophage prostaglandins by the EFAD diet may allow for an increase in the release of IL-2 and IL-4. Enhanced IL-4 production by T cells from EFAD-treated NOD mice, as shown in this study, could positively influence the activation of T cells, in particular Th2 cells, and influence the incidence of diabetes (13).

We also evaluated the production of IL-1 and TNF-α by LPS-stimulated macrophages and found that the production of both of these cytokines was significantly increased by the EFAD diet (see Fig. 6). Previous studies have demonstrated that the production of IL-1 and TNF-α is limited in NOD macrophages (54,55). The increase in the production of IL-1 and TNF-α may be explained by the reduction in macrophage eicosanoid metabolism, as prostaglandins suppress the production of these cytokines (56,57). In addition, enhanced TNF-α release may act to suppress the production of prostaglandins and therefore be additive or perhaps mediate the effect of the EFAD diet on eicosanoid production (58). The augmented production of IL-1 by macrophages, coupled with the reduced prostaglandin production, may allow for a more efficient activation of Th2 cells. Together, these results demonstrate functional changes in macrophages that may contribute to reduced suppression and improved activation of T cells by this APC population.

It may be questioned whether a reduced intake of nutrients could have influenced diabetes incidence in the EFAD group. Studies performed in diabetes-prone BB rats, using calorically restricted control rats, have shown that the protective effect of EFAD was not a function of decreased weight (3). Moreover, it should also be emphasized that NOD mice and C57BL/6 in our study exhibited a strikingly different susceptibility toward the growth retarding effect of EFAD. Indeed, C57BL/6 mice were reported to be among the most sensitive inbred mouse strains to dietary obesity (59). Our observations may also indicate a genetic predisposition for this nutritional trait.

In summary, our data indicate that the manipulation of dietary fatty acids is able to prevent the onset of diabetes in the NOD mouse model and suggest that the effect is mediated through functional changes in both T cells and APC, particularly macrophages. The profound changes in the activation of T cells by mitogens, and in the increased production of IL-4, suggest that the activity of T cells, in particular Th2 cells, is markedly affected. Changes in the functional characteristics of macrophages may also contribute to the enhanced activity of the T cell population. Our findings provide new insights into the mechanisms of the dietary effects of EFAD on the immune response and the development of autoimmune diabetes.

Acknowledgment: This work was supported by a grant from the Nora Eccles Treadwell Foundation. P.Y.B. was the recipient of a grant from French Ministère des Affaires Etrangères (Bourse Lavoisier). The authors thank Ms. D. Bagga for performing fatty acid measurements.

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