PREVENTION OF DIABETES IN THE BB RAT BY ESSENTIAL FATTY ACID DEFICIENCY

Relationship between Physiological and Biochemical Changes

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Essential fatty acid (EFA)[1] deficiency is remarkably protective against immune-mediated renal disease. Mortality from murine lupus, due largely to immune-mediated glomerulonephritis, is substantially attenuated by the deficiency state (1). We have observed a similar beneficial effect in immune-mediated glomerulonephritis using the model of nephrotoxic nephritis in rats (2). The influx of macrophages into the glomerulus in this model is completely prevented by EFA deficiency, and subsequent proteinuria (an indicator of the renal injury) is decreased ~90%. Studies on interstitial nephritis induced by the administration of puromycin aminonucleoside have also established that EFA deficiency prevents the influx of macrophages into the renal interstitium and concomitantly prevents expected reductions in glomerular filtration and renal blood flow (3). A protective effect of EFA deficiency is also seen in the interstitial inflammation that accompanies experimental hydronephrosis (4).

Recently, we have shown that such protective effects are not limited to renal diseases. Using the low dose streptozotocin-treated mouse model of autoimmune diabetes, we observed that EFA deficiency completely prevents both pancreatic insulitis and hyperglycemia (5). To extend our understanding of these protective effects, the present experiments were undertaken to study the influence of EFA deficiency on diabetes in the BB rat.

Diabetes-prone (DP)-BB rats develop a heritable, ketosis-prone diabetes syndrome similar to human insulin-dependent diabetes mellitus, and many lines of evidence suggest an autoimmune pathogenesis of diabetes in both disorders (6–8). Islet autoantibodies are present (9), the pancreatic islets of diabetic rats are infiltrated by

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[1] *Abbreviations used in this paper:* DP, diabetes prone; DR, diabetes resistant; EFA, essential fatty acid.

lymphocytes (an inflammatory lesion termed insulitis), and adoptive transfer diabetes has been demonstrated (10, 11). The DP-BB rat also has severe T cell lymphopenia (12) with reduced numbers of $CD4^+$ and $CD8^+$ T cells and complete absence of $RT6^+$ T cells (13, 14). Several immunosuppressive interventions, including cyclosporine (15), thymectomy (16), antilymphocyte serum (17), silica (18, 19), and total lymphoid irradiation (20), prevent diabetes in the BB rat. In addition, at least one immunomodulatory intervention, lymphocyte transfusion, prevents both diabetes and insulitis (21, 22).

The diabetes-resistant (DR)-BB rat was derived from DP forebears but bred for resistance to the disease (7, 23). It has a cumulative incidence of diabetes of <1%. In contrast to DPs, DR-BB rats have normal numbers and a normal distribution of all lymphocyte phenotypes, including $RT6^+$ cells. The rare DR animals that do become diabetic are not lymphopenic (24). However, in vivo immune elimination of $RT6^+$ T lymphocytes in DR rats by administration of anti-RT6.1 lymphotoxic antibody precipitates diabetes in ~50% of 30-d-old animals within 3–4 wk (25). Cells expressing the RT6 antigen in the rat (~50% of $CD4^+$ and ~70% $CD8^+$ T lymphocytes) are believed to exert a regulatory influence on the immune system, and the RT6-depleted DR-BB rat is used as a model of inducible autoimmune diabetes (26).

In the present study, we show that EFA deficiency protects against diabetes in both DP and RT6-depleted DR animals. The degree of protection, however, was found to be somewhat variable. This variability provided us with an opportunity to examine the relationship between this physiological effect of EFA deficiency and its biochemical effects.

EFA deficiency is characterized by depletion of essential (n-6) polyunsaturated fatty acids and accumulation of (n-9) polyunsaturated fatty acids. Linoleate and its desaturation/elongation product, arachidonate, are both depleted (27). In lieu of these fatty acids, oleate accumulates and is desaturated and elongated into the fatty acid 20:3(n-9), which is normally not synthesized and whose accumulation serves to define the deficiency state (27). Consequently, EFA deficiency is not only a deficiency state, but is also a condition characterized by accumulation of an abnormal fatty acid, 20:3(n-9), which, like arachidonate, can be metabolized to a variety of potentially active biological mediators (28–30). Interpreting the effects of EFA deficiency has heretofore been problematic in that there has been no prima facie evidence as to whether any biological effect is due to the depletion of (n-6) fatty acids (or their metabolites) or the accumulation of (n-9) fatty acids (or their metabolites). In the present study, however, the response to EFA deficiency exhibited some variability, and we were thus able to correlate the physiological effect of the deficiency state directly with its biochemical effects. The results suggest that depletion of (n-6) fatty acids may be the key biochemical variable affected by EFA deficiency.

Materials and Methods

Animals. BB rats were obtained from the University of Massachusetts, Worcester. Animals from this colony are designated BB/Wor. The cumulative incidence of disease in the DP line of BB/Wor rats is 40–70%; >85% of all cases appear between 60 and 120 d of age; <0.5% appear before 60 d of age (7). Both sexes are equally susceptible. The cumulative incidence of diabetes among DR-BB/Wor rats is <1% among >20,000 animals studied.

Except where noted, rats in the present studies were 22–30 d old when entered into experiments, were housed under standard laboratory conditions, given ad libitum access to food and water, and were weighed and tested for glycosuria (Tes-Tape; Eli Lilly, Inc., Indianapolis, IN) twice weekly. Diabetes was diagnosed on the basis of glycosuria and a plasma glucose concentration >250 mg/dl, as determined by a glucose analyzer (Beckman Instruments, Inc., Fullerton, CA).

Reagents and Materials. Polyunsaturated fatty acid methyl esters for gas chromatography standards were purchased from Sigma Chemical Co. (St. Louis, MO), except for 20:3(n-9) methyl ester, which was purchased from Biomol (Plymouth Meeting, PA). Silica gel plates for the isolation of fatty acid methyl esters (20 × 20 cm, 0.25 mm) were purchased from Analabs (North Haven, CT). Chemicals were purchased from Burdick & Jackson Laboratories Inc. (Muskegon, MI) and were HPLC grade.

Dietary Studies. Two diets were used in this study. The control diet consisted of standard Purina 5001 Rodent Laboratory Chow. The EFA-deficient diet was purchased from Purina Test Diets (Richmond, IN) and contained casein (vitamin free, 21%), sucrose (68.85%), dl-methionine (0.15%), non-nutritive fiber (3%), vitamin mixture (2%), choline chloride (0.2%), and mineral mixture (5%). The EFA-deficient diet was periodically monitored for fatty acid content and consistently contained <10 μg of linoleate per gram of food and no detectable arachidonate. Animals (22–25 d of age) were starved for 24 h to augment the depletion of essential fatty acids, and then were begun on the EFA-deficient diet. All experiments were performed using both male and female rats.

Experiment 1 was carried out with DP-BB rats in two separate trials. In the first trial, DP animals were maintained on either the control or EFA-deficient diet through 120 d of age or until they became diabetic. Animals were killed when they became diabetic or at 120 d of age, and samples of liver and pancreas were obtained. Livers were frozen at −20°C for later lipid analysis (see below). Pancreata were fixed in Bouin's solution, embedded in paraffin, stained with hematoxylin and eosin, and studied for the presence of insulitis by light microscopy. The second trial in Experiment 1 was identical except that the rats were studied through 200 d of age.

Experiment 2 was carried out using the RT6-depleted DR-BB rat model. Newly weaned littermates, 22 d of age, were fasted for 24 h and then randomized to receive either the control or EFA-deficient diet. 7 d later, they began treatment with DS4.23 anti-RT6.1 antibody (25). The antibody was injected intraperitoneally, 2 ml/rat, five times/wk, in the form of supernatants obtained from primary cultures maintained in our laboratory. Injections were continued for 4 wk. Animals were killed when they developed diabetes, or between 62 and 71 d of age. Lymphocyte subset analysis was performed using standard flow microfluorimetry methods, as previously described (7), on a sample of rats in both groups to confirm depletion of $RT6^+$ T cells. The experiment was performed as three separate trials as litters of rats became available. Pancreatic histology was performed on the nondiabetic rats at the end of the third trial only.

To control for the previously recognized (27) growth retardation that occurs in EFA-depleted animals, a third experiment was performed in which male and female littermate DP rats were randomized to receive either Purina rat chow ad libitum or a diet limited to 6 g of the same chow daily. The amount of chow given to the food-restricted group had previously been determined to be the average amount consumed by 40-d-old DP-BB/Wor rats. Both experimental and control animals were weighed and tested for diabetes through 120 d of age. They were then killed and not studied further.

The fourth experiment investigated the effect of linoleate repletion in BB rats previously made EFA deficient. As in the first experiment, DP-BB rats were randomized to the EFA-deficient or the control diet. Beginning at 70 d of age, half of the EFA-deficient rats were begun on linoleate given as the free acid (Sigma Chemical Co.), 1 ml/rat three times weekly by orogastric gavage, until the animals were 163 d of age. In a second trial, an identical protocol was used except that linoleate repletion was begun at 120 d of age and continued through 156 d. Control animals were not gavaged.

Lipid Analysis. Lipids from samples of liver were extracted by the method of Bligh and Dyer (31). Fatty acids were then transmethylated by the sequential addition of 0.5 N NaOH in methanol and 6 N HCl. Fatty acid methyl esters were extracted with hexane/diethyl ether,

1:1, and separated by thin layer chromatography (hexane/diethyl ether/acetic acid, 75:5:1). Fatty acid methyl esters were identified by comigration with authentic standard, the appropriate band was scraped, and the methyl esters were eluted from the plate with hexane/diethyl ether, 1:1.

Fatty acid methyl esters were characterized and quantified by gas chromatography using a gas chromatograph (5890) interfaced with an integrator (3393A; Hewlett-Packard Co., Palo Alto, CA). The column used was an SP-2380 capillary column (0.32-mm diameter, 30-m length) from Supelco, Inc. (Houston, TX), and was run at 170°C isothermally. The injector was maintained at 250°C and the flame ionization detector at 300°C. Peaks were identified by comigration with authentic standards and quantified by the integrator. Results are expressed as mole percent.

Statistical Analysis. Nonparametric data are presented as $2 \times N$ tables and were analyzed using either the Fisher exact statistic or the χ^2 statistic. Parametric data are for the most part presented as mean ± SEM, and were analyzed using the unpaired t test or one-way analysis of variance. Data on the relationship of the change in fatty acids and the onset of diabetes were analyzed by linear regression to obtain correlation coefficients and p values. A total of 12 animals that died acutely during gavage with linoleate have been excluded from all analyses; details are given in the text. A very small number of animals found dead in their cages have also been excluded from analysis.

Results

Effects of EFA Deficiency on the Diabetes and Insulitis in the DP-BB Rat. We examined the effect of the deficiency state on the frequency of spontaneous diabetes in the DP rat in two independent trials carried out through 120 and 200 d of age, respectively. As shown in Table I, EFA deficiency substantially decreased the incidence of disease and delayed its onset in both trials. In the first trial, EFA deficiency markedly reduced the incidence of diabetes but did not completely prevent its occurrence. The cumulative incidence of diabetes in EFA-deficient animals was 29%, as compared with 80% in controls ($p < 0.005$). When the occurrence of insulitis, the pathological substrate of autoimmune diabetes, was taken into account, the relative protective effect of the EFA-deficient state was still apparent; 100% of controls were affected with either diabetes or insulitis, as compared with only half of rats on the EFA-deficient diet ($p < 0.0005$). The mean age at onset of diabetes was somewhat greater in EFA-deficient animals (104 ± 5 d) compared with controls (98 ± 4 d), but this difference did not reach statistical significance. The body weight of nondiabetic rats at 90 d of age (the mean age at which diabetes generally occurs in DP rats) is also indicated in Table I, and shows the mild growth-retarding effect of the EFA-deficient state, which averaged 56 g for males and 18 g for females.

The occurrence of insulitis in several nondiabetic EFA-deficient rats and the trend toward later onset among diabetic EFA-deficient animals suggested the possibility that the diet had delayed rather than prevented diabetes. This possibility was addressed in the second trial of Experiment 1, which extended the treatment period to 200 d of age. It confirmed the observations reached in the first trial. As shown in Table I, EFA deficiency again reduced (to 35%), but did not completely block, the occurrence of diabetes (which reached 65% in controls, $p < 0.05$). In this trial, we did observe that EFA deficiency also delayed the mean age of onset of diabetes: 121 ± 6 d of age for the EFA-deficient group vs. 104 ± 4 d of age for controls ($p < 0.05$). Unfortunately, technically inadequate and therefore uninterpretable histology on six of the seven nondiabetic control pancreata precluded a comparative analysis of the frequency of insulitis. We can report, however, that insulitis was found

TABLE I

Effect of EFA Depletion on the Frequency of Diabetes and Insulitis in the DP-BB Rat

Diet	Trial	No. with diabetes	Mean age at onset of diabetes	No. of nondiabetics with insulitis	No. with diabetes or insulitis	Body weight at 90 d of age		Body weight at 150 d of age	
						Male	Female	Male	Female
			d			*g*		*g*	
EFAD	1	6/21 (29%)*	104 ± 5‡	4/15 (27%)	10 (48%)	258 ± 10 (n = 8)	198 ± 7 (n = 9)		
	2	6/20 (30%)‖	121 ± 6¶			287 ± 10 (n = 8)	207 ± 4 (n = 9)	319 ± 11 (n = 7)	227 ± 6 (n = 6)
Control	1	16/20 (80%)	98 ± 4	4/4 (100%)	20 (100%)	314 ± 8 (n = 8)	216 ± 8 (n = 6)		
	2	13/20 (65%)	104 ± 4			374 ± 6 (n = 6)	202 ± 20 (n = 3)	436 ± 12 (n = 4)	231 ± 16 (n = 3)

Experimental animals were begun on the EFA-deficient diet after a 24-h fast. EFAD, EFA deficiency.

* $p < 0.0005$ vs. control group.

‡ $t = 0.79$, p = NS vs. control.

§ $p < 0.05$ vs. control group.

‖ $p < 0.0005$ vs. control group.

¶ $t = 2.47$, $p = 0.05$ vs. controls.

in only 5 of the 14 pancreata (36%) obtained from 200-d-old nondiabetic EFA-depleted rats. Thus, this second trial confirmed that EFA deficiency prevented, and did not simply delay, diabetes in the DP-BB rat.

Effect of EFA Deficiency on the Frequency of Diabetes in RT6-depleted DR-BB Rats. Typically, about half of DR-BB rats treated with a cytotoxic antibody directed against $RT6^+$ lymphocytes develop diabetes (25). Using this model, EFA deficiency was even more strikingly protective. As shown in Table II, 28% of RT6-depleted DR controls (n = 29) developed diabetes, whereas no antibody-treated EFA-deficient animals (n = 26) developed diabetes. Lymphocyte subset analysis confirmed that anti-RT6 antibody depleted $RT6^+$ T cells in the EFA-deficient animals to the same degree as in the controls (data not shown).

EFA deficiency was also associated with the absence of insulitis in nondiabetic animals in the third trial of Experiment 2. Among 12 EFA-deficient rats in this trial, none developed either diabetes or insulitis. Among 13 controls, three became diabetic and three of the nondiabetic pancreata showed insulitis. Considering insulitis and diabetes together, the difference between the two groups was statistically significant at the p <0.02 level.

Effect of Linoleate Repletion of EFA-deficient BB Rats. To determine if the protective effect of EFA deficiency was specifically due to deprivation of (n-6) fatty acids, EFA-deficient DP-BB rats were repleted with linoleate at two time points. Repletion was begun either at 70 d of age (the age by which diabetes has begun to appear in control animals) or at 120 d of age (after the peak incidence of spontaneous diabetes in DP rats). These two experiments also addressed whether the protective effect of the deficiency state was absolutely dependent on the altered fatty acid composition or whether a more permanent alteration in susceptibility could be effected.

Linoleate repletion of EFA-deficient DP animals at 70 d of age appeared to restore their susceptibility to diabetes (Table III). The incidence of diabetes through 163 d of age in repleted EFA-deficient animals was intermediate between control and EFA-deficient animals, and was not significantly different from either, although there was a trend towards a difference from EFA-deficient animals. The effect of linoleate repletion was more apparent when the frequency of insulitis was taken into account. Ei-

TABLE II

Effect of EFA Depletion on the Frequency of Diabetes and Insulitis in the RT6-depleted DR-BB Rat

	Overall results		Results of trial 3			
Diet	No. with diabetes	Mean age at onset of diabetes	No. with diabetes	No. of diabetics studied histologically	No. of nondiabetics with insulitis	No. with diabetes or insulitis
		d				
EFAD	0/26 (0%)*	-	0/12 (0%)	12	0/12 (0%)‡	0/12 (0%)§
Control	8/29 (28%)	60 ± 2	3/13 (23%)	10	3/10 (30%)	6/13 (46%)

The study was carried out in three trials as litters became available. Histologic study was included as part of the third trial only. Depletion of $RT6^+$ T cell populations to undetectable (<5%) levels was confirmed in a random sampling of rats from both the experimental and control groups. The animals tested histologically were selected from among littermates randomized to the two conditions as part of the third trial.

* $p < 0.01$ vs. controls.

‡ $p < 0.09$ vs. controls.

§ $p < 0.005$ vs. controls.

TABLE III

Effect of EFA Depletion and Subsequent Linoleate Repletion on the Frequency of Diabetes and Insulitis in the DP-BB Rat

Diet	No. with diabetes	Mean age at onset of diabetes	No. of nondiabetics with insulitis	No. with diabetes or insulitis
		d		
EFAD	3/16 (19%)*	136 ± 5	3/13 (23%)	6/16 (38%)‡
EFAD and linoleate	6/11 (55%)	106 ± 7	4/5 (80%)	10/11 (91%)
Control	14/17 (82%)	105 ± 7	1/3 (33%)	15/17 (88%)

A total of 33 experimental rats were begun on the EFA-deficient diet at 22 d of age after a 24-h fast. At 70 d of age, a total of 17 of these animals were selected at random and begun on a regimen of linoleate repletion (1 ml/rat, three times weekly) by gavage. Six of these animals died acutely during the administration of linoleate and have been excluded from the analysis. All rats were tested for diabetes through 163 d of age.

* Overall $\chi^2 = 13.40$, $df = 2$, $p < 0.01$; $p < 0.001$ vs. control diet group; no other paired comparisons are significant.

‡ Overall $\chi^2 = 13.14$, $df = 2$, $p < 0.01$; $p < 0.01$ vs. both the linoleate repleted group and the control diet group; no other paired comparisons are significant. There were no statistically significant differences in the age at onset of diabetes ($F = 2.54$, p = NS).

ther diabetes or insulitis was observed in nearly all controls (as seen above), as well as in most repleted EFA-deficient animals. In contrast, 63% of EFA-deficient animals (10/16) were free of both diabetes and its pathological substrate, insulitis.

The study of linoleate repletion begun at 120 d of age was carried out to completion in 11 controls, but only six EFA-deficient animals; six additional EFA-deficient rats died acutely while being gavaged between 130 and 156 d of age. Through 120 d of age, 5 of the 11 control DP rats, but none of the 12 EFA-deficient animals, became diabetic. Between 120 and 156 d of age, no additional control animals became diabetic, nor did diabetes appear in any of the six surviving linoleate-repleted EFA-deficient rats. Histologic study of the six pancreata from these EFA-deficient, linoleate-repleted rats revealed insulitis in only one. Parenthetically, we note also that none of the six rats that died acutely during linoleate gavage (between 10 and 36 d after its institution) were diabetic ante mortem.

Relationship of Body Weight and Diabetes Frequency. Because EFA deficiency is known to cause modest growth retardation, the question arose as to whether the protective effect of the deficiency state might be due simply to a decrease in body weight. As was expected on the basis of previous experience, EFA-deficient rats grew at a slower rate than did controls (Table I). However, it was observed in the third experiment that simple starvation resulting in an even greater degree of growth retardation was not associated with any reduction in the frequency of diabetes (Table IV).

Additional information bearing on this issue was obtained from the linoleate repletion experiments. As was the case in Experiment 1 (Table I), EFA deficiency led to a 10–30% decrease in body weight in both male and female animals. Repletion of EFA-deficient animals with linoleate at 70 d of age, however, did not lead to a significant gain in weight in repleted animals (data not shown). Nonetheless, as described above, repleted animals exhibited a degree of susceptibility to diabetes similar to that of control animals, suggesting that simple growth retardation was not sufficient to decrease the incidence of diabetes.

TABLE IV

Effect of Caloric Restriction on the Frequency of Diabetes in Male and Female DP-BB Rats

Sex	Diet	Body weight ~60 d	Body weight ~90 d	Body weight ~115 d	No. with diabetes	Percent	Mean age at onset
		g					*d*
Male	ad libitum	231 ± 11 (n = 12)	303 ± 8 (n = 10)	347 ± 12 (n = 4)	8/12	75	97 ± 5
Male	6 gm/d	98 ± 2 (n = 9)	107 ± 5 (n = 6)	119 ± 5 (n = 4)	5/9	56	84 ± 5
Female	ad libitum	168 ± 5 (n = 9)	216 ± 12 (n = 8)	233 ± 16 (n = 6)	3/9	33	89 ± 5
Female	6 gm/d	88 ± 2 (n = 11)	99 ± 5 (n = 7)	112 ± 5 (n = 5)	6/11	55	82 ± 4

Body weights are given for rats at three different ages: 60 d, when the first cases of diabetes appear; 90 d, when the incidence of diabetes is maximal; and 115 d, when most cases of diabetes have already appeared. Ages when weights were measured are approximate (± 3 d) due to variation in date of birth. All calorie-restricted rats were begun on this regimen at 30 d of age. The amount of food given these rats was based on the average amount consumed by a group of 40-d-old DP rats. The number of animals at each successive data point declines due to the elimination of diabetic rats from further study. All rats were studied through 120 d of age. There are no statistically significant differences in the frequency of diabetes or its age at onset among any of the four groups.

Relationship between the Physiological and Biochemical Effects of EFA Deficiency. As shown in Table V, EFA deficiency led to marked decreases in hepatic (n-6) fatty acids (i.e., linoleate and arachidonate), as well as to the accumulation of (n-9) fatty acids, oleate and 20:3(n-9), in the DP-BB rats that were part of Experiment 1. The aggregate 20:3(n-9) to arachidonate ratio of EFA-deficient animals was 3.0, well above the 0.4 ratio used as the biochemical definition of the deficiency state (27). Because the protective effect of the deficiency state was not universal in our initial experiment, we sought to determine if the degree of deficiency might correlate with disease susceptibility. The hepatic fatty acid analysis of diabetic and nondiabetic EFA-deficient animals,

TABLE V

Hepatic Fatty Acid Analysis of DP-BB Rats

Experimental group	Fatty acid 18:1(n-9)	18:2(n-6)	20:3(n-9)	20:4(n-6)	20:3(n-9)/20:4(n-6)
Controls (n = 4)	12.3 ± 0.3	14.2 ± 1.0	-	13.2 ± 1.9	-
EFAD (n = 17)	35.5 ± 1.8*	2.2 ± 0.7*	8.2 ± 0.8*	4.2 ± 0.8*	3.0 ± 0.4*
EFAD + linoleate (n = 7)	29.3 ± 1.9*	4.4 ± 1.2*	0.4 ± 0.1*	5.0 ± 1.2*	0.1 ± 0.0‡

Liver lipids from DP-BB rats were extracted and the constituent fatty acids transmethylated for analysis by gas chromatography as detailed in Materials and Methods. Quantitation of the major (n-9) and (n-6) polyunsaturated fatty acids are shown together with the 20:3(n-9) to arachidonate ratio. 18:1(n-9), 18:2(n-6), and 20:4(n-6) represent oleic, linoleic, and arachidonic acids, respectively. Results are expressed as mole percent.

* $p < 0.01$, EFAD or EFAD plus linoleate vs. controls.

‡ $p < 0.05$, EFAD plus linoleate vs. controls.

as an index of their response to the diet, was therefore compared. As shown in Table VI, diabetic EFA-deficient animals in general exhibited less marked changes in fatty acids. This association appeared particularly strong for the (n-6) fatty acids. EFA-deficient animals that developed diabetes tended to have more nearly normal levels of linoleate and arachidonate than did the nondiabetic EFA-deficient animals. Interestingly, although EFA-deficient animals that developed diabetes had lower levels of oleate, their levels of 20:3(n-9) were no different from those of nondiabetic animals.

To strengthen this association, we next sought to determine if, in the group of EFA-deficient animals that did become diabetic, there was a correlation between age at onset of diabetes with changes in the major polyunsaturated fatty acids. As shown in Fig. 1, the age at onset of diabetes in this subgroup showed a significant negative correlation with the level of both linoleate and arachidonate. The more depleted of (n-6) fatty acids an animal was, the longer it was resistant to the development of disease. With respect to the (n-9) fatty acids, there was a significant positive correlation with the level of oleate, but there was no apparent correlation with the level of 20:3(n-9). Thus, these data corroborate those presented above, in that the degree of change in fatty acids, particularly (n-6) fatty acids, appeared to be related to disease susceptibility.

We subsequently examined the effect of linoleate repletion on fatty acid composition and the propensity to develop diabetes. As shown in Table V, linoleate repletion at 70 d of age potently suppressed levels of 20:3(n-9) and decreased levels of oleate. Linoleate and arachidonate levels were only modestly affected. Nonetheless, the 20:3(n-9) to arachidonate ratio of these animals was in the normal range. When linoleate-repleted EFA-deficient animals were divided into diabetic and nondiabetic subgroups, the only apparent difference in fatty acid composition was in the level of arachidonate (Table VI). Arachidonate levels in diabetic animals were more nearly normal than in nondiabetic animals. Thus, disease susceptibility in these experiments correlated most closely with the level of arachidonate.

TABLE VI

Hepatic Fatty Acid Analysis of EFA-deficient and Linoleate-supplemented EFA-deficient DP-BB Rats: Diabetics vs. Nondiabetics

	Fatty acid				20:3(n-9)
Experimental group	18:1(n-9)	18:2(n-6)	20:3(n-9)	20:4(n-6)	20:4(n-6)
EFAD					
Nondiabetic (n = 9)	40.5 ± 0.9	0.9 ± 0.2	7.5 ± 0.8	2.0 ± 0.3	4.2 ± 0.4
Diabetic (n = 8)	30.1 ± 2.7*	3.6 ± 1.3‡	9.1 ± 1.5	6.7 ± 1.2*	1.7 ± 0.4*
EFAD + linoleate					
Nondiabetic (n = 3)	31.5 ± 1.8	3.1 ± 1.7	0.4 ± 0.1	2.9 ± 0.6	0.2 ± 0.0
Diabetic (n = 4)	26.3 ± 3.5	6.0 ± 1.3	0.4 ± 0.1	7.8 ± 1.5‡	0.1 ± 0.0

Liver lipids from EFAD- and EFAD-linoleate-supplemented DP rats were extracted and the constituent fatty acids transmethylated and analyzed by gas chromatography as detailed in Materials and Methods. Quantitation of the major (n-9) and (n-6) polyunsaturated fatty acids are shown along with the 20:3(n-9) to arachidonate ratio. 18:1(n-9), 18:2(n-6), and 20:4(n-6) represent oleic, linoleic, and arachidonic acids, respectively. Results are expressed as mole percent. Animals were divided into nondiabetic and diabetic groups for comparison.

* $p < 0.01$, nondiabetic vs. diabetic.

‡ $p < 0.05$, nondiabetic vs. diabetic.

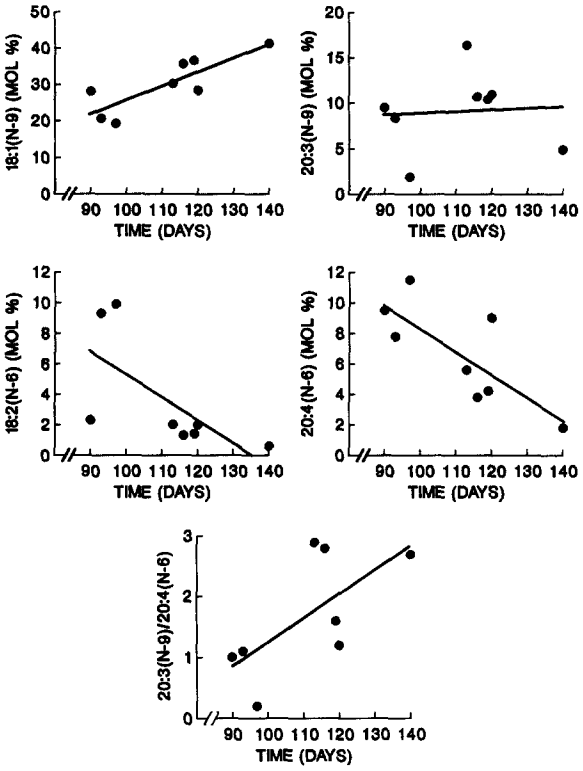

FIGURE 1. Regression analysis of hepatic fatty acid content on time of onset of diabetes in DP-BB rats. Hepatic fatty acid composition was determined in diabetic DP-BB rats by gas chromatography as detailed Materials and Methods. The levels of the major (n-9) and (n-6) polyunsaturated fatty acids and the 20:3(n-9) to arachidonate ratio were correlated with the time of onset of diabetes. 18:1(n-9), 18:2(n-6), and 20:4(n-6) represent oleic, linoleic, and arachidonic acids, respectively. Correlation coefficients and p values are: oleic acid, $r = 0.84$, $p < 0.05$; 20:3(n-9), $r = 0.07$, p = NS; linoleic acid, $r = 0.67$, $p < 0.05$; arachidonic acid, $r = 0.76$, $p < 0.05$; 20:3(n-9) to arachidonate ratio, $r = 0.66$, $p < 0.05$.

Discussion

Previous studies have provided strong evidence that diabetes in the BB rat is an autoimmune process (6, 7). As noted above, lymphocytes from acutely diabetic DP-BB rats adoptively transfer the disease, and various immunosuppressive regimens ameliorate it. In the DR-BB rat, diabetes is induced by in vivo immune elimination of its $RT6^+$ T cells. The present data indicate clearly that an environmental manipulation (EFA depletion) can prevent diabetes in both DP and RT6-depleted DR-BB rats.

The fact that only about half of inbred DP or RT6-depleted DR rats become diabetic has suggested to many investigators that there may be a role for environmental perturbants in this disorder. In previous studies of the BB rat, however, the potential importance of environmental factors, such as diet, hormonal state, stress, and infection, in this disease model has been unclear. The appearance of diabetes in BB rats reared in a gnotobiotic environment has excluded horizontal but not vertical transmission of an infectious agent (32), yet the incidence of diabetes is reportedly reduced by infection with lymphocytic choriomeningitis virus (33). Hormonal manipulations such as hypophysectomy and castration, pancreatic parasympathetic denervation by vagotomy, agents that protect against β cell injury (3-*O*-methyl glucose and niacinamide), and stress have all been investigated in the BB rat and found not to affect disease incidence (34). We have previously reported that diets high in

carbohydrate, fat, or protein were also ineffective in preventing diabetes (34), but other investigators report that semisynthetic diets based on casein or utilizing L-amino acids reduce the frequency of diabetes, whereas those based on wheat gluten increase it (35, 36). Diets containing omega-3 fatty acids (fish oil) also influence the frequency of diabetes (37). The physiological and biochemical basis of the protective effect exerted by dietary manipulations in the BB rat have to date remained obscure.

In previous studies, we observed that the dietary manipulation of EFA deficiency is remarkably protective in certain models of autoimmune disease. Recently, we established that EFA deficiency completely prevents the insulitis and diabetes in the low dose streptozotocin-induced model of diabetes (5). This study provided the impetus to investigate the effects of EFA deficiency on diabetes in the BB rat. The present study extends our prior observations and establishes the protective effect of the deficiency state in both the spontaneously diabetic DP-BB rat and the RT6-depleted DR-BB rat. In these models, EFA deficiency markedly decreased the incidence of diabetes and insulitis. In addition, it moderately delayed the onset of the disease in EFA-deficient DP animals that nonetheless did become diabetic. The protective effect of EFA deficiency was reversed by selective supplementation of DP-BB rats with linoleate at 70 d of age, but not when linoleate was begun at 120 d of age. These results confirm and extend previous suggestions (38) that DP rats are susceptible to immunomodulation of diabetes only during a critical period that extends to ~2 mo of age. If protected during this vulnerable period, they will not subsequently develop diabetes.

The beneficial effect of EFA deficiency did not appear to be a simple function of a decrease in body weight. Linoleate-supplemented EFA-deficient BB rats were nearly identical in weight to EFA-deficient animals, but exhibited a degree of susceptibility to diabetes comparable with that of control animals. These results are corroborated by our independent result showing that caloric restriction and body weight, per se, are not determinants of the expression of diabetes in the BB rat.

Although EFA deficiency was virtually completely protective in the low-dose streptozotocin model in mice in our previous studies (5), the deficiency state was not as absolutely protective in the present studies on the DP-BB rat. This could be in part due to the fact that diabetes develops between 60 and 120 d of age in this model of diabetes. Since it takes weeks to make animals fully EFA deficient (27), insulitis in these animals may be developing at a time when the animals are incompletely EFA deficient. In our prior studies using the low-dose streptozotocin model in mice, it was possible to make animals fully EFA deficient before the induction of disease (5).

Because the response to this dietary manipulation was not absolute in the DP-BB rat, we were afforded the unique opportunity to examine the relationship between the fatty acid changes induced by EFA deprivation with its physiological effects. These experiments suggest that the depletion component of the EFA deficiency state, rather than the accumulation of (n-9) fatty acids, particularly the abnormal 20:3(n-9), is of paramount importance in diabetes prevention. In particular, the depletion of the (n-6) fatty acid, arachidonate, appeared to correlate closely with disease susceptibility. This observation is not an obvious consequence of EFA deficiency. Although it is well appreciated that EFA deficiency may lead to a decrease in arachidonate with alterations in the production of prostaglandins, thromboxanes, and leukotrienes (39, 40), it is less well known that 20:3(n-9), like arachidonate, can be metabolized

to a variety of eicosanoids that are potentially bioactive (28–30). Consequently, it has heretofore not been apparent biochemically how EFA deficiency exerts its effects.

The exact mechanisms underlying the protective effect of EFA deficiency, however, remain to be fully elucidated. It appears that the effects of EFA deficiency are not mediated through gross alterations in humoral or cellular immunity, as these immunologic functions are basically intact in EFA-deficient animals (41). Two alternative (and not mutually exclusive) explanations for the beneficial effects of the deficiency state in autoimmune disease are under consideration. First, there could be a lack of a critical lipid mediator (e.g., arachidonate or a metabolite derived therefrom) involved in the initiation of the immune injury. Alternatively, there could be a decrease in the influx of macrophages into the focus of insulitis.

Regarding the first possibility, studies have shown a decrease in the generation of lipid mediators such as leukotriene B_4 (40) and platelet-activating factor (42) with EFA deficiency. Both of these substances may act to attract leukocytes to a focus of inflammation (43, 44). It is currently unknown, however, whether islets can synthesize either of these mediators.

The potential importance of macrophages to diabetes in the BB rat has recently been highlighted by studies showing that macrophages are an early constituent of the insulitis in this model (19). In addition, it has been shown that the administration of silica, a crude macrophage toxin, prevents diabetes in the DP-BB rat (18, 19, 45). Furthermore, studies suggest that certain cytokines of macrophage origin may inhibit β cell function and lead to β cell destruction (46). The hypothesis that the beneficial effects of the deficiency state may be mediated through its effects on macrophages is supported by recent studies on glomerulonephritis. In normal animals, the glomerulus is populated with resident macrophages (47). These cells are absent from the glomerulus in EFA deficiency and return with supplementation of (n-6), but not (n-3), fatty acids (47). In the context of acute glomerulonephritis, leukocytes invade the glomerulus; polymorphonuclear neutrophils predominate early on followed by macrophages (2). EFA deficiency selectively prevents the influx of macrophages in this model of tissue injury (2).

In conclusion, EFA deficiency significantly reduces the frequency of diabetes in both the DP and in the RT6-depleted DR-BB/Wor rat. These effects appear to be the consequence of the depletion of arachidonate. We would conjecture that a lipid mediator (possibly a metabolite of arachidonate) and macrophages are key elements in the development of insulitis in this model. EFA deficiency would appear to exert its protective effect through alterations of arachidonate metabolism, as well as on macrophage function and/or migration. Hopefully, further understanding of the mechanisms underlying the beneficial effects of EFA deficiency will lead to the development of new strategies to understand and to prevent autoimmune diabetes.

Summary

Essential fatty acid (EFA) deficiency exerts a striking protective effect in several animal models of autoimmune disease. We now report that EFA deprivation prevents diabetes in the BB rat, an animal model of human insulin-dependent diabetes mellitus. In diabetes-prone (DP)-BB rats, the incidences of spontaneous diabetes and insulitis (the pathological substrate of autoimmune diabetes) were greatly re-

duced by EFA deficiency. This beneficial effect of the deficiency state was also seen in diabetes-resistant (DR)-BB rats that, after treatment with antibody to eliminate $RT6^+$ T cells, would otherwise have become diabetic. The susceptibility of EFA-deprived DP-BB rats to spontaneous diabetes was restored when they were given dietary supplements of linoleate at 70 d of age (during the usual period of susceptibility), but not when they were repleted beginning at 120 d (after the peak incidence of diabetes). EFA deficiency did lead to growth retardation, but calorically restricted control rats demonstrated that the protective effect of the deficiency state was not a function of decreased weight. To examine the relationship between the biochemical changes of EFA deficiency and its physiological effects in this system, we compared the fatty acid changes that occurred in EFA-deficient animals that did and did not develop diabetes. Nondiabetic animals had significantly lower levels of (n-6) fatty acids (i.e., linoleate and arachidonate) and higher levels of oleate, an (n-9) fatty acid, than did diabetic animals. Levels of 20:3(n-9), the fatty acid that uniquely characterizes EFA deficiency, were similar in both groups, however. Among diabetic EFA-deficient rats, the age at onset of diabetes was found to correlate inversely with the level of (n-6) fatty acids, the least depleted animals becoming diabetic earliest, whereas there was no correlation with levels of 20:3(n-9). Among animals repleted with linoleate beginning at 70 d, restoration of susceptibility to diabetes correlated with normalization of the level of arachidonate. In summary, EFA deprivation reduced the frequency of diabetes in both DP and RT6-depleted DR-BB rats. This protective effect was strongly associated with depletion of (n-6) fatty acids, particularly arachidonate, but not with accumulation of the abnormal 20:3(n-9). Conjecturally, arachidonate and/or a metabolite may play a key role in mediating inflammatory injury in this animal model of autoimmune diabetes.

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