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Essential fatty acid deficiency prevents multiple low-dose streptozotocin-induced diabetes in naive and cyclosporin-treated low-responder murine strains

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Abstract. We have previously shown that essential fatty acid (EFA) deficiency prevents diabetes and ameliorates insulitis in low-dose streptozotocin (LDS)-treated male CD-1 mice. The effects of EFA deficiency on the incidence of diabetes after LDS treatment has not been examined in other strains. In contrast to highly susceptible CD-1 mice, several other strains of mice are only partially susceptible to LDS treatment and do not develop appreciable insulitis; however, the susceptibility of these strains can be markedly increased by cyclosporin A (CsA) pretreatment to reduce suppressor cell function. Weanling male BALB/cByJ, DBA/2J, and C57BL/6J mice were placed on EFA-deficient (EFAD) or control diets for 2 months and then divided into experimental and control groups. Ten EFAD and 10 control mice from each strain received LDS treatment (40 mg/kg/d 5 d); an additional 10 EFAD BALB/cByJ and another 10 control BALB/cByJ mice received subcutaneous CsA injections (20 mg/kg/d) for 14 days prior to and for 5 days simultaneous with LDS treatment (40 mg/kg/d 5 d). Plasma glucose levels for all mice were determined 3 times per week for 3 weeks after LDS treatment. Mean plasma glucose levels (±SEM) at the end of the experiment were significantly lower in the EFAD groups vs control groups in BALB/cByJ ($P<0.001$), DBA/2J ($P<0.00001$), and C57BL/6J ($P=0.012$) mice. CsA supplementation increased the severity of diabetes in LDS-treated BALB/cByJ mice ($P<0.0005$); however, EFA deficiency also prevented diabetes in CsA-supplemented BALB/cByJ mice. Peri-insulitis was seen in 50% of control vs 40% of EFAD DBA/2J (NS) and in 20% of control vs 0% of EFAD C57BL/6J (NS) mice. Insulitis was not seen in either of these strains. Insulitis or peri-insulitis was seen in 10% of EFAD, 25% of EFAD+CsA, 33% of control, and 80% of control+CsA BALB/cByJ mice ($P<0.05$). We conclude that EFA deficiency prevents LDS-induced diabetes in all three of these partially susceptible strains as well as in BALB/cByJ mice augmented with CsA. EFA deficiency was confirmed biochemically on plasma samples.

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Key words: Streptozotocin – Diabetes – Insulitis – Autoimmunity – Essential fatty acids

Introduction

Streptozotocin (STZ), a methylnitrosourea (MNU) with a 2-substituted glucose, is a pancreatic beta-cell toxin that has been widely used to induce experimental diabetes mellitus. When given in a single, large, intravenous dose, it induces direct beta-cell necrosis within 24–48 h. In 1976, Like and Rossini reported that multiple 'subdiabetogenic' doses of STZ incite a lymphocytic insulitis and diabetes in male CD-1 mice [1]. The onset of hyperglycaemia requires several weeks. The presence of an autoimmune component is evidenced not only by lymphocytic infiltration into the islets and the thymic dependency of the diabetic state, but by the ability to ameliorate or prevent the diabetes with immunosuppression [2–4].

Few other mouse strains respond to LDS like the CD 1 mouse; most are resistant or only partially susceptible [2–4]. Shortly after the original description of the LDS model, Rossini et al. [5] reported that some mouse strains showed a moderate sensitivity to the diabetogenic effects of LDS but did not develop lymphocytic insulitis (or only very minimal insulitis). In these mice, diabetes developed over a period of several weeks rather than over several days as might be expected if merely due to the cumulative cytotoxic effects of LDS. Leiter's group made similar observations when they examined multiple inbred strains, including *scid/scid* mice who have no functional T or B lymphocytes but have normal numbers of macrophages, granulocytes and NK cells [2, 6].

Initially, resistance vs susceptibility was thought to be determined entirely on a genetic basis. However, later evidence showed that resistant strains can be made susceptible by selective immunosuppression, probably directed at suppressor T cells. Both cyclophosphamide at a dose of 70 mg/kg i.p. either 1 or 6 days before LDS [7] and cyclosporin A (CsA) at a dose of 20 mg/kg/d subcutaneously

for 2 weeks prior to and simultaneous with the LDS treatments [8] have been reported to induce susceptibility to both insulitis and diabetes in resistant strains. Likewise, CsA treatment has also been shown to increase the severity of insulitis and diabetes in LDS-treated CD-1 mice [9, 10].

We have previously demonstrated that essential fatty acid (EFA) deficiency prevents insulitis and diabetes in male CD-1 mice given LDS [11, 12]. In that study, essentially all of 19 LDS-treated mice on control chow developed diabetes, with mean plasma glucose levels of about 22.2 mM (400 mg/dl). At autopsy, the mice had severe insulitis, with lymphocytic infiltration into the islets and beta cell necrosis. Only 1/13 mice on the EFA deficient (EFAD) diet developed even mild diabetes; the mean plasma glucose level for this group was about 7.2 mM (130 mg/dl). In LDS-treated EFAD mice, there was focal, mild peri-insulitis with negligible beta-cell degranulation.

In the present study, we examined whether EFA deficiency would prevent diabetes in strains that are weakly sensitive to LDS and whether any protective effect would persist when the LDS treatment was augmented with CsA pretreatment. BALB/cByJ, in contrast to the strongly resistant BALB/cJ mice, are partially sensitive to the diabetogenic effects of LDS but do not develop appreciable insulitis [6]. Likewise, C57BL/6J and DBA/2J mice are also partially susceptible to the diabetogenic effects of LDS; the former do not develop appreciable insulitis, while the latter develop a milder insulitis than that seen in LDS-treated CD-1 mice [5].

Materials and methods

All studies were performed with the approval of the Dalhousie University Committee on Laboratory Animal Care in accordance with the principles laid down by the Canadian Council on Animal Care. Weanling male BALB/cByJ, C57BL/6J and DBA/2J mice (Jackson Lab, Bar Harbor, Maine, USA) were placed on EFA-deficient (Purina 5803C low essential fatty acid purified diet, Purina Test Diets, Richmond, Ind., USA) or control (Agway Prolab 3000, Syracuse, N.Y., USA) chow for 2 months as in previous studies [11, 12]. Ten EFAD and 10 control mice from each strain received LDS (UpJohn, Kalamazoo, Mich., USA) treatment (40 mg/kg/d i.p. for 5 days); an additional 10 EFAD and 10 control BALB/cByJ mice received subcutaneous CsA (Sandimmune IV, Sandoz, Montreal, Canada) injections (20 mg/kg/d) for 14 days prior to and for 5 days simultaneous with LDS treatment. Mice were bled retro-orbitally and tested for hyperglycaemia 3 times per week after LDS treatment. Plasma glucose levels were determined using a Beckman glucose analyser II (Fullerton, Calif., USA). Diabetes was defined as non-fasted plasma glucose levels >11.1 mM (200 mg/dl).

All mice were killed on day 26 and their pancreases processed for microscopy. H&E-stained sections were examined blindly and were graded as showing no mononuclear cell infiltrates, peri-insulitis [i.e. lymphocytic infiltrate at the ductal pole of islet(s) and/or surrounding but not invading islet(s)] or insulitis [i.e. lymphocytic infiltrate invading islet(s)]. Slides showing either peri-insulitis or insulitis were ranked blindly in order of severity. Histological data were examined by the rank-sum test as described below.

The presence or absence of EFA deficiency was confirmed biochemically in the BALB/cByJ mice. Three random plasma samples from each of the four groups of mice were collected when the mice were killed. The fatty acids contained in these samples were derivatized to methyl esters by the method of Moser and Moser and were then analysed by gas chromatography as in previous studies [12–14]. Briefly, aliquots of fatty acid methyl esters suspended in carbon disulphide were injected on a 30×0.25 mm fused silica column coated with SP-2330 (Supelco, Oakville, Ontario, Canada). The methyl esters were separated by running a Hewlett-Packard gas chromatograph (model 5890 II) isothermally at 195°C. Injector and detector temperatures were 250°C. The column head pressure was 14 kPa, the linear flow rate was 22 cm/s, and the split ratio was 30:1. The signal from the flame ionization detector was collected and analysed using a Maxima 820 Chromatography Workstation (Millipore-Waters, Mississauga, Ontario, Canada). EFA deficiency was defined as ratio of Mead acid, [20:3(n-9)], to arachidonate exceeding 0.4 [15].

Statistical analyses were performed on an Acer Acros 4865X/25 personal computer. Analysis of variance followed by paired comparisons was performed with Statfast Statistical Supplement software (Statsoft, Tulsa, OK), and Wilcoxon's rank sum test and *t*-test (two-tailed) were performed with Epistat statistical software (Tracy L. Gustafson, M.D., Round Rock, Texas).

Results

Table 1 shows the mean serum fatty acid profiles of CsA-treated and untreated BALB/cByJ mice on control or EFAD diets. The ratio of Mead acid [20:3(n-9)] to arachidonate [20:4(n-6)] greatly exceeded 0.4, thus confirming severe EFA deficiency [15] in both EFAD and EFAD+CsA groups.

Figure 1 shows the mean plasma glucose levels in control and EFAD DBA/2J and C57BL/6J mice. The mean plasma glucose level prior to LDS treatment for EFAD DBA/2J, control DBA/2J, EFAD C57BL/6J and control C57BL/6J mice was 8.3, 7.6, 8.8 and 7.4 mM, respectively. At the end of the experiment, these levels were compared by *t*-tests and were significantly lower in both EFAD groups (Fig. 1). Peri-insulitis was seen in 50% of control vs 40% of EFAD DBA/2J mice (NS) as well as 20% of control vs 0% of EFAD C57BL/6J (NS) mice. Full-blown insulitis was not seen in any DBA/2J or C57BL/6J mouse. The incidence of diabetes during the final week in EFAD and control DBA/2J mice was 10% and 100%, respectively; the final incidence in EFAD and control C57BL/6J mice was 30% and 80%, respectively.

Figure 2 shows mean plasma glucose levels in the four groups of BALB/cByJ mice throughout the study. Mean plasma glucose levels for EFAD and control BALB/cByJ mice were 5.8 and 6.8 mM, respectively, prior to CsA treatment; after 14 days of CsA treatment (i.e. immediately prior to the first LDS treatment), the mean plasma glucose level in EFAD, EFAD+CsA, control and control+CsA mice was 5.8, 4.9, 6.2 and 7.6 mM, respectively. At the

Table 1. Fatty acid analysis of plasma from CsA-treated and untreated BALB/CByJ mice on control and EFAD diets

	n	20:3 (n-9) (wt. %)	20:4 (n-6) (wt. %)
EFAD	3	7.7±1.9	0.4±0.3
EFAD + CsA	3	13.7±0.8	0.7±0.4
Control	3	1.8±0.1	8.7±0.6
Control + CsA	3	1.9±0.2	9.4±0.6

Values are reported as means±SEM
EFAD, Essential fatty acid deficient; CsA, cyclosporin A

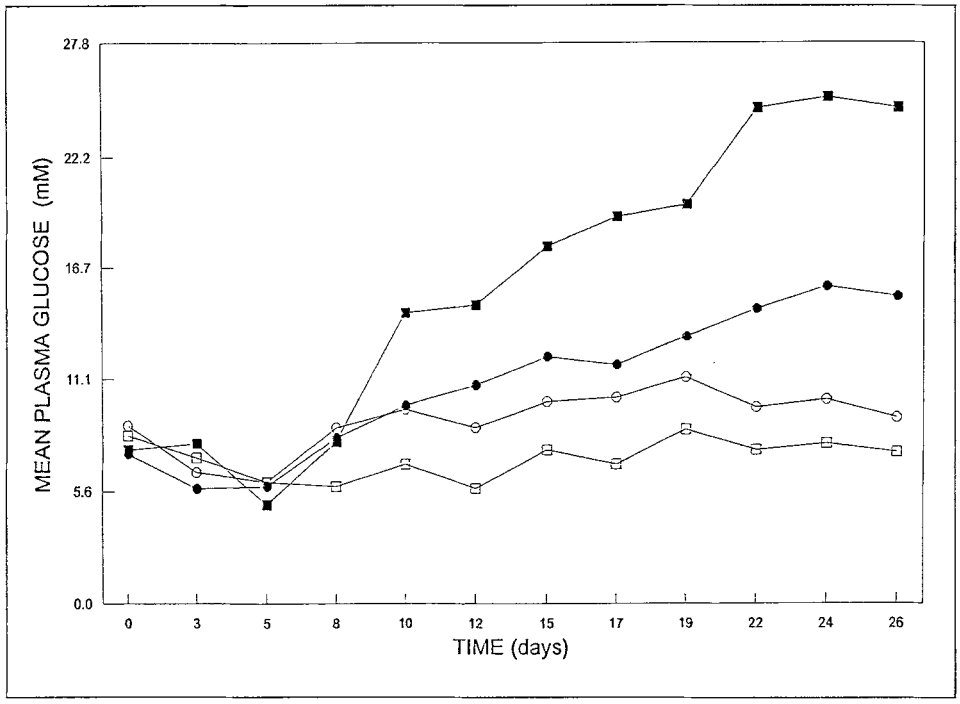

Fig. 1. Daily mean plasma glucose levels in LDS-treated EFAD and control DBA/2J and C57BL/6J mice. At the end of week 4 *t*-tests were performed to compare control and EFAD DBA/2J (t=9.83; df=17; P<0.00001) as well as control and EFAD C57BL/6J mice (t= 2.79; df=18; P=0.012). ■ Control DBA/2J; ⊟ EFAD, DBA/2J; ⊖ EFAD, C57BL/6J; ● Control C57BL6J

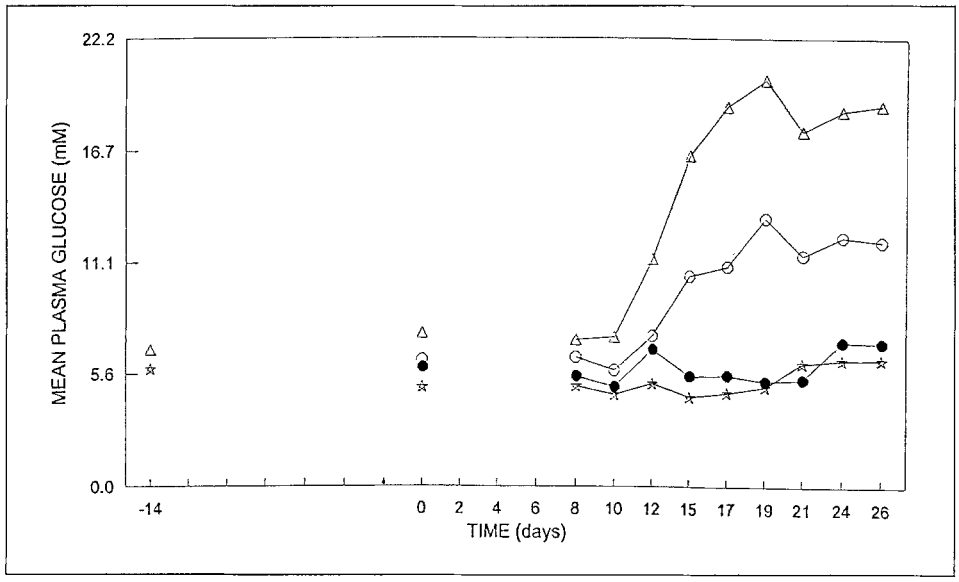

Fig. 2. Daily mean plasma glucose levels in LDS-treated EFAD and control BALB/cByJ mice as well as in LDS-treated, CsA-pretreated EFAD and control BALB/cByJ mice. Analysis of variance (ANOVA) was performed to compare means at the end of week 4. ANOVA $F(3,33)$=34.2; P<0.0001. Paired comparisons: control vs EFAD: t=3.57, df=33, P<0.001; control vs CsA-control: t=4.81, df=33, P<0.0005; CsA-control vs CsA-EFAD: t=8.69, df=33, P<0.0005; EFAD vs CsA-EFAD: t=0.56, df=33, NS. ● BALB/cByJ EFAD, only LDS (40 mg/kg i.p.); ☆ BALB/cByJ EFAD, CsA (20 mg/kg s.c.)+LDS (40 mg/kg i.p.); △ BALB/cByJ Control, CsA (20 mg/kg s.c.)+LDS (40 mg/kg i.p.); ⊖ BALB/cByJ control, only LDS (40 mg/kg i.p.)

end of the experiment, the mean plasma glucose level (±SEM) in EFAD, EFAD+CsA, control and control+CsA mice was 7.1±0.5, 6.3±0.4, 12.2±0.9 and 18.9±1.5 mM, respectively. Terminal plasma glucose levels for the four groups were compared by analysis of variance [$F(3, 33)$=34.2; P<0.0001] followed by paired comparisons. Two important observations are apparent. First, EFA deficiency protected both naive (P<0.001) and CsA-treated (P<0.0005) BALB/cByJ mice from the diabetogenic effects of LDS. Second, CsA treatment increased the terminal mean plasma glucose levels in control mice

Table 2. Grading of insular lymphocytic infiltrates in LDS-treated BALB/cByJ mice

	n	Normal	Peri-insulitis	Insulitis
EFAD	10	90%	10%	0%
EFAD + CsA	8	75%	25%	0%
Control	9	67%	22%	11%
Control + CsA	10	20%	50%	30%

Chi-square (df=3)=8.88, P<0.05 (rank-sum test)
LDS, Low-dose streptozotocin

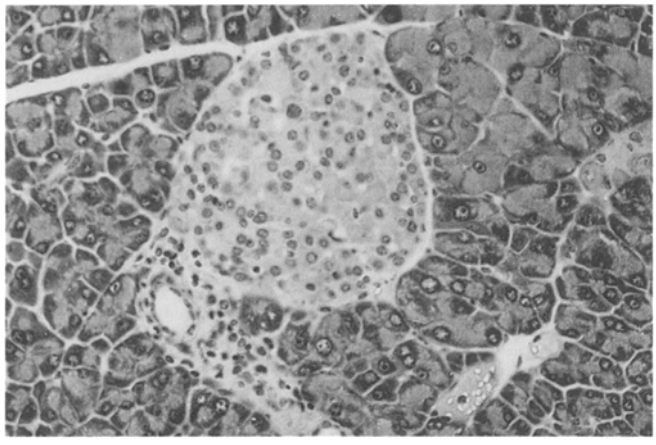

Fig. 3. Section showing very mild peri-insulitis characterized by a scanty periductal mononuclear cell infiltrate at the ductal pole of the islet (H&E stain, ×300)

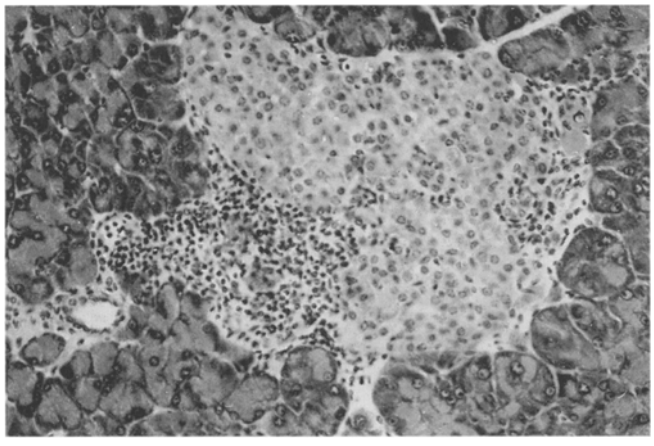

Fig. 4. Section showing insulitis characterized by mononuclear cells infiltrating the islet (H&E stain, ×200)

($P<0.0005$) but not in EFAD mice. The incidence of diabetes during the final week in EFAD, EFAD+CsA, control and control+CsA mice was 0%, 0%, 67% and 100%, respectively.

Table 2 shows the incidence of insulitis and peri-insulitis in each of the four groups of BALB/cByJ mice. CsA treatment increased the incidence of insular lesions, especially in control chow-fed mice. Only one-third of LDS-treated control mice developed either insulitis or peri-insulitis, while 80% of CsA-treated control mice developed these lesions ($P<0.05$) (Figs. 3 and 4).

Discussion

We have previously shown that EFA deficiency prevents diabetes and greatly diminishes lymphocytic insulitis in LDS-treated CD-1 mice, a highly susceptible strain [11, 12]. The finding that LDS treatment prevents diabetes even in partially susceptible strains which do not develop significant lymphocytic insulitis suggests that the protective effect of EFA deficiency cannot be directed at the lymphocytic insulitis. This is an important observation that sheds light on the mechanism of both LDS diabetes and EFA deficiency's protective effect.

In a previous study [12], we hypothesised that full-blown LDS insulitis and diabetes require five distinct steps: (1) LDS-induced beta cell 'damage' causing neoantigen expression; (2) influx of activated macrophages ("single cell insulitis") into the damaged islet, amplifying the damage by free radical and cytokine (IL-1, TNF, etc.) production; (3) processing and presentation of beta-cell fragments by activated macrophages; (4) activation of T helper cells; and (5) lymphocytic infiltration. In a susceptible strain like CD-1 mice, all of these steps occur, resulting in full expression of the insulitis and severe hyperglycaemia. In partially susceptible strains, it is likely that only the first three steps occur. However, when selective immunosuppression is used to depress suppressor cell function, even less susceptible strains progress through all five steps, resulting in full-blown insulitis and diabetes. In the absence of selective immunosuppression, many of the LDS-treated mice from these less susceptible strains will retain a sufficient beta-cell mass to eventually regenerate minimally adequate insulin supplies [2]. In contrast, strains with a strong lymphocytic response (e.g. CD-1 or C57BL/KsJ) almost invariably develop permanent, severe diabetes [2].

We believe that the insulitis is a result of the adaptive immune system's recognition of the LDS-modified islets. This is further evidenced by the observation that islet isografts performed 2 weeks after the first LDS treatment do not incite a lymphocytic infiltrate even though insulitis is still progressing in the native islets of the pancreas [16, 17]; on the other hand, Lacy and Weide [17] have recently demonstrated that islets treated in vitro with LDS were destroyed by insulitis when isografted into LDS-treated diabetic mice with active insulitis. Therefore, it seems likely that the lymphocytic immune response is only directed at LDS-modified islets.

Studies combining EFA deficiency and LDS treatment permit insights into the mechanism of LDS insulitis and diabetes. Even though the nature of the LDS-induced neoantigen is unknown, EFA deficiency clearly does not prevent its formation since EFA repletion by injecting 99% pure methyl linoleate 3 days after LDS precipitates a 100% incidence of insulitis and diabetes [11]. However, the window of susceptibility during which EFA supplementation will initiate the destruction of a LDS-modified beta-cell is only a few weeks [12]. The observation that immediate repletion causes diabetes and that delayed repletion does not also suggests that EFA deficiency's protective effect is exerted prior to T-cell involvement (i.e. steps 4 and 5) since presumably immune "memory" should be invoked after that.

We believe that the protective effect of EFA deficiency probably acts at the level of the macrophage by preventing the 'single cell insulitis' that occurs prior to lymphocytic insulitis in LDS-treated mice [18]. Because EFA deficiency markedly diminishes leukotriene B4 production and inhibits the influx of leucocytes in response to inflammatory stimuli [19, 20], EFA deficiency should tend to

minimize the influx of macrophages (i.e. the "single cell insulitis") into LDS-damaged islets. In the absence of EFA deficiency, it seems likely that the initial LDS damage is amplified by the influx of activated macrophages. Islets are very sensitive to free radical damage, in part, because they possess low levels of free radical scavenging enzymes [21, 22]. Therefore, when these activated macrophages release free radicals, more extensive beta-cell damage would occur, which attracts more activated macrophages, resulting in a cascade effect. This amplification scheme would also explain our observation that the hydroxyl radical scavenger desferrioxamine, administered beginning 5 h after the last LDS injection, prevents LDS-induced insulitis and diabetes [23]. In addition to functioning as sources of oxygen radicals, activated macrophages also release monokines such as IL-1 and TNF which are cytotoxic to islets [24]; these could further amplify the beta-cell damage.

The presence of mouse strains that are partially sensitive to the diabetogenic effect of LDS in the absence of a significant lymphocytic insulitis suggests that the "single cell insulitis" alone is diabetogenic for some strains. In the present study, we have shown that EFA deficiency prevents LDS diabetes in all three partially sensitive strains tested: BALB/cByJ, C57BL/6J and DBA/2J mice. The observation that EFA deficiency protects these partially sensitive strains suggests that the protective effect is exerted at step 2, the "single cell insulitis". EFA deficiency, by blocking the influx of macrophages into LDS-damaged islets, prevents the amplification of the damage which we believe causes the milder diabetes seen in less susceptible strains.

In conclusion, EFA deficiency is highly protective in various experimental models for autoimmune diabetes. Several groups have now shown that EFA deficiency prevents or ameliorates diabetes, insulitis and even thyroiditis in spontaneously diabetic BB Wistar rats [25–27]. We have previously shown that EFA deficiency prevents LDS-induced insulitis and diabetes in male CD-1 mice, a strain that is highly susceptible to LDS. In the present study, we have shown that EFA deficiency also prevents LDS-induced diabetes in all three partially sensitive strains tested and that this protective effect persists even when LDS is augmented with CsA treatment. On the other hand, EFA deficiency does not protect mice from the direct toxicity of STZ. We have previously shown that EFAD mice given a single intravenous injection of "high-dose" STZ became severely diabetic. We believe that EFA deficiency's protective effect is due to an inhibition of "single cell insulitis". The highly protective effect of macrophage-directed therapy in the LDS model has also been demonstrated by Oschilewski et al. [28].

To date, no one has been able to determine whether EFA deficiency is protective in spontaneously diabetic NOD mice. We attempted such a study but were forced to terminate it because of the long time course required for the onset of spontaneous diabetes in NOD mice and because of the toxic effects of very long-term EFA deficiency. Following this, we attempted to determine whether EFA deficiency would prevent diabetes in cyclophosphamide-treated NOD mice (i.e. reducing suppressor cell function), because this treatment greatly accelerates the onset of diabetes in NOD mice [29]; unfortunately, the combination of EFA deficiency and cyclophosphamide treatment was uniformly fatal (unpublished observation). Both of these studies highlight a major problem associated with EFA deficiency, i.e. its chronic toxicity. Even though the chronic toxicity of EFA deficiency prevents its use clinically in prediabetic children, this experimental model continues to provide interesting insights into the mechanism of autoimmune diabetes.

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