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Essential fatty acid deficiency prevents multiple low-dose streptozotocin-induced diabetes in CD-1 mice

(insulitis/autoimmunity/linoleate/eicosanoids/macrophages)

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ABSTRACT **Multiple i.p. injections of low-dose streptozotocin (40 mg/kg) produce insulitis, beta cell destruction, and diabetes in male CD-1 mice. Recent data also suggest that macrophages figure in the low-dose streptozotocin model. Because other recent studies have shown that essential fatty acid deficiency prevents autoimmune nephritis in mice, decreases the number of resident Ia-positive glomerular macrophages, and decreases the elicitation of macrophages into the glomerulus in inflammation, we examined the effect of essential fatty acid deficiency on the incidence and severity of insulitis and diabetes in CD-1 mice treated with low-dose streptozotocin. Streptozotocin-treated mice on the control diet uniformly developed diabetes (19/19). Essential fatty acid-deficient mice treated with streptozotocin did not develop diabetes (1/13). Mean plasma glucose levels for the control and essential fatty acid-deficient mice were 384.5 ± 23.6 and 129.1 ± 15.5 mg/dl, respectively, at the end of 1 month. To discern whether essential fatty acid deficiency prevented the streptozotocin-induced beta cell injury or the inflammatory response to injured beta cells, mice were repleted with daily injections of 99% pure methyl linoleate beginning 3 days after the last streptozotocin injection. These mice also quickly developed severe (3/4) or mild (1/4) diabetes. Histologic examination of the pancreata of control mice or repleted mice showed marked insulitis and beta cell destruction; in contrast, the pancreata of essential fatty acid-deficient mice showed preservation of beta cells and only focal mild peri-insulitis. Essential fatty acid deficiency thus prevents the insulitis and resultant diabetes in low-dose streptozotocin-treated CD-1 mice, suggesting a central role for macrophages and lipid mediators in this autoimmunity model.**

Type 1 diabetes mellitus in man is believed to be a multifactoral disease involving genetic, environmental, and autoimmune components (for reviews, see refs. 1–3). The multiple low-dose streptozotocin mouse, an animal model of type 1 (juvenile-onset) diabetes, shares these characteristics. Streptozotocin, a methylnitrosourea with a 2-substituted glucose, is a drug widely used to induce experimental diabetes (for review, ref. 4). When the drug is given in one large dose, beta cell necrosis and severe diabetes occur in the absence of significant inflammation within 48 hr. In 1976, Like and Rossini reported that multiple subdiabetogenic doses of streptozotocin result, instead, in a lymphocytic infiltration into the islets (i.e., insulitis) followed by a more gradual onset of severe diabetes (5). In this model, susceptibility to insulitis is in part genetic in that it is associated with the mouse major histocompatibility complex (6). An autoimmune component is documented by several studies demonstrating that immunosuppressive regimens ameliorate or prevent diabetes (7–10). Ultrastructural studies have suggested a key role for the macrophage in the streptozotocin-induced insulitis, for this inflammatory cell is the first to be detected within the islet after streptozotocin administration (11). Data showing that silica, a macrophage toxin, prevents diabetes in this model supports this contention (12–14).

Recent evidence established that dietary polyunsaturated fatty acid modulation exerts a marked protective effect in autoimmune disease. This effect is most striking in the context of murine lupus, where both essential fatty acid deficiency [i.e., deprivation of (n-6) fatty acids such as 18:2(9,12) or 20:4(5,8,11,14) where (n-6) indicates the location of the terminal double bond to the end of the molecule] as well as (n-3) [i.e., fatty acids such as 20:5(5,8,11,14,17)] fatty acid enrichment, prevent inception of the immune-mediated glomerulonephritis and the consequent mortality (15–19). Studies from our laboratory into mechanisms underlying this effect suggested this beneficial effect to be due to the effects of polyunsaturated-fatty acid modification on resident macrophages. Essential fatty acid deficiency has decreased numbers of resident macrophages in the glomerulus (20) and peritoneum (21) as well as their generation of chemotactic factors, such as leukotriene B_4 (22). Both these effects may contribute to decreased leukocyte movement into foci of inflammation (21).

In this study, we probed the role of the macrophage in streptozotocin-induced insulitis using essential fatty acid deficiency. Results establish that essential fatty acid deficiency is strikingly protective in this model of type 1 diabetes mellitus and suggest that the macrophage is a pivotal cell in the insulitis.

METHODS AND MATERIALS

Male VAF$^+$ (virus antibody free) weanling CD-1 mice (21 days old) were purchased from Charles River Breeding Laboratories, housed five per cage, and fed either standard laboratory animal chow (Purina 5001, Saint Louis, MO) or a chow deficient in essential fatty acids (Purina 5803C low essential fatty acid purified diet, Purina Test Diets, Richmond, IN) ad libitum for 2–3 mo. Purina 5803C is a casein hydrolysate (21.00%) supplemented with sucrose (68.65%), solka floc (3.00%), choline chloride (0.20%), minerals (5.00%), vitamins (2.00%), and DL-methionine (0.15%). After 2–3 mo on either diet, essential fatty acid deficient (n = 14) and control (n = 20) mice were retroorbitally bled using heparinized capillary tubes, and plasma was collected to validate the deficiency state (see below). After confirming essential fatty acid deficiency, mice were weighed and ear-notched for identification. Streptozotocin (0070C lot from Upjohn) was dissolved in citrate buffer and immediately

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Table 1. Fatty acid analysis of plasma from CD-1 mice on control and essential fatty acid-deficient diets

	n	18:1(9), mol %	18:2(9,12), mol %	20:3(5,8,11), mol %	20:4(5,8,11,14), mol %
Control	3	16.0 ± 0.5	19.5 ± 2.5	0	3.4 ± 1.2
EFAD	3	53.1 ± 0.3	0	11.0 ± 1.1	0
EFAD + 18:2	3	39.9 ± 5.2	6.0 ± 0.8	1.0 ± 0.5	1.8 ± 0.1

18:1(9) and 20:3(5,8,11) are (n-9) fatty acids, whereas 18:2(9,12) and 20:4(5,8,11,14) are (n-6) fatty acids. EFAD, essential fatty acid-deficient. Values are reported as means ± SEM.

administered i.p. at a dose of 40 mg per kg of body weight per day for 5 days (5).

All mice were tested for hyperglycemia three times per week. Plasma glucose concentrations were determined by the glucose oxidase method using a glucose analyzer (Beckman Instruments, Fullerton, CA). Diabetes was defined as non-fasted plasma glucose values >200 mg/dl.

At the end of the fourth week, all animals were sacrificed after the last plasma glucose determination, and the pancreata were removed, fixed in Bouins' solution, and processed for light microscopy. Sections of pancreas were stained either with hematoxylin/eosin or with aldehyde fuschin, a stain for insulin-containing beta cell granules.

Linoleate Repletion. Studies were also done to determine the effect of linoleic acid repletion on the incidence of diabetes in streptozotocin-treated essential fatty acid-deficient mice. Four essential fatty acid-deficient CD-1 mice were given i.p. injections of 99% pure linoleic acid methyl ester (Sigma) at a dose of 50 μl/day (i.e., ≈1 g per kg/day) for 5 days per week beginning on day 8 (i.e., 3 days after the last streptozotocin injection). These mice were monitored for hyperglycemia and insulitis as above. Plasma was obtained at the end of the experiment for fatty acid analyses.

Fatty Acid Analyses. Fatty acid analyses were performed on plasma samples as described (23). Briefly, the lipids were extracted from plasma samples by the method of Bligh and Dyer (24). Fatty acid methyl esters were formed by the addition of 10% BF_3 in methanol at 56°C for 45 min, extracted with hexane, and purified by one-dimensional thin-layer chromatography (hexane/anhydrous diethyl ether/acetic acid, 75:5:1 vol/vol). Fatty acid methyl esters were identified and measured on a Varian 3700 gas chromatograph interfaced with a Varian 4270 integrator using a glass column (6 ft × 4 mm) packed with 10% SP-2340 on 100/120 Chromasorb (Supelco, Houston, TX). The nitrogen flow rate was 40 ml/min, and the injector and flame ionization detector were maintained at 280°C. Comigration with authentic standards (Supelco) identified individual fatty acid methyl esters.

Determination of Number of Ia-Positive Cells per Islet. Islets were isolated from untreated control and essential fatty acid-deficient mice by the collagenase method (25), separated on a discontinuous Ficoll gradient (26), and picked with a Pasteur pipette while being visualized through a dissecting microscope. Freshly isolated islets were stained for Ia antigens by indirect immunofluorescence. Because CD-1 mice are an outbred strain, we used a mixture of Ia antibodies containing A.TH anti-A.TL (stains mouse Ia specificities 1, 2, 3, 7, 15, 19, 22, 31, and 32; from V. Hauptfeld, Washington University School of Medicine, Department of Genetics), OX-3 (cross-reacts with mouse Ia specificity 9, Accurate Chemicals, Westbury, NY), and OX-4 (cross-reacts with mouse Ia specificities 17 or 18, Accurate Chemical) as the primary antibodies; goat anti-mouse IgG-fluorescein isothiocyanate (Tago, Burlingame, CA) was used as the secondary antibody. After staining, 50 control and 50 essential fatty acid-deficient islets were examined under a fluorescent microscope, and numbers of Ia-positive cells per islet were recorded. Additional islets stained with the secondary antibody alone exhibited no labeling.

RESULTS

Validation of Essential Fatty Acid Deficiency. Essential fatty acid deficiency was verified in mice before the experiment by plasma fatty acid analysis. Table 1 shows that plasma from essential fatty acid-deficient mice exhibited a marked depletion of linoleate [18:2(9,12)] and arachidonate [20:4(5,8,11,14)] [i.e., the principal (n-6) fatty acids] and an increase in oleate [18:1(9) and 20:3(5,8,11)] [i.e., the principal (n-9) fatty acids]. As noted, the ratio of 20:3(5,8,11) to arachidonate exceeded 0.4, the biochemical definition of essential fatty acid deficiency (27).

Effect of Essential Fatty Acid Deficiency on Streptozotocin-Induced Diabetes. Fig. 1 *Upper* shows the incidence of diabetes in streptozotocin-treated essential fatty acid-deficient and control CD-1 mice at 2- to 3-day intervals during the 4 weeks of the experiment. After day 15, virtually 100% of the control mice were diabetic; in contrast, <10% of the essential fatty acid-deficient mice were diabetic. Two essential fatty acid-deficient mice experienced transient hyperglycemia but returned to normoglycemia (one mouse had a plasma glucose level of 204 mg/dl on day 19; the other had levels of 209 and 237 mg/dl on days 17 and 22, respectively); a third essential fatty acid-deficient mouse sustained a relatively persistent mild diabetes (plasma glucose levels between 188 and 244 mg/dl after day 12).

Fig. 1 *Lower* shows the mean plasma glucose levels for streptozotocin-treated essential fatty acid-deficient and control mice at 2- to 3-day intervals. Control mice exhibited severe diabetes with a mean blood glucose of ≈400 mg/dl.

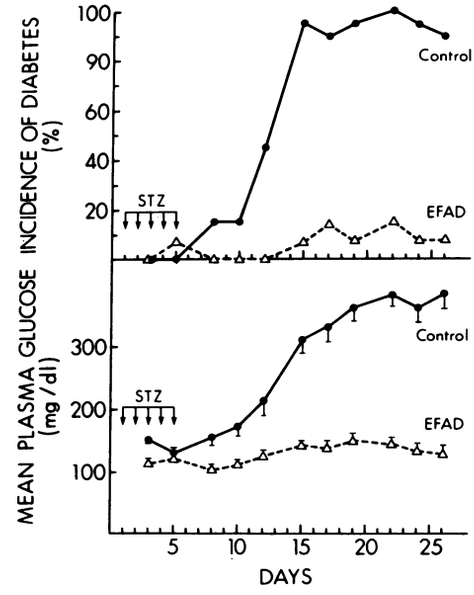

FIG. 1. (*Upper*) Daily incidence of diabetes (plasma glucose >200 mg/dl) in 13 or 14 essential fatty-acid deficient (EFAD) mice (one nondiabetic essential fatty acid-deficient mouse died on day 19) and in 19 or 20 control CD-1 mice (one diabetic control mouse died on day 17) treated with five i.p. injections of streptozotocin (STZ) at 40 mg/kg. (*Lower*) Corresponding daily mean plasma glucose levels.

However, mean plasma glucose levels in the essential fatty acid-deficient mice at the end of the experiment were within the normal range and were one-third that of the control mice.

Effect of Essential Fatty Acid Deficiency on Insulitis. Because essential fatty acid deficiency protected against the low-dose streptozotocin-induced diabetes, we examined the effect of the deficiency state on the incidence and severity of insulitis in these mice. Fig. 2 shows the typical histologic appearance of islets from streptozotocin-treated control and streptozotocin-treated essential fatty acid-deficient CD-1 mice. Sections of pancreas from streptozotocin-treated control mice frequently showed massive lymphocytic infiltrates surrounding and infiltrating the islets (Fig. 2*B*), especially in mice with partially granulated islets and plasma glucose levels <400 mg/dl. Control mice with plasma glucose levels >400 mg/dl had little ongoing insulitis, as this process subsides after most beta cells are destroyed. In these mice, the islets were small and were composed predominantly of nonbeta cells. Sections from streptozotocin-treated essential fatty acid-deficient CD-1 mice revealed only minimal beta cell degranulation and several islets per pancreas with peri-insulitis (i.e., lymphocytic infiltrates either at the periductal pole of the islet or surrounding but not invading the islet) (Fig. 2*C*). The most prominent lesion in most islets was dilatation of the periductal lymphatics by lymphocytes. Frank insulitis (i.e., lymphocytic infiltration) with disruption of the islet architectural integrity was not seen.

Effect of Linoleate Repletion. To determine whether essential fatty acid deficiency prevented the initial streptozotocin-induced injury or the subsequent expression of the inflammatory response, we repleted streptozotocin-treated essential fatty acid-deficient mice with linoleate 3 days after the final streptozotocin injection. Linoleate supplementation led to decreases in (n-9) fatty acids and increases in (n-6) fatty acids (Table 1). Three of the four repleted mice became diabetic within 2 weeks of the first injection of linoleic acid (plasma glucose levels between 350 and 650 mg/dl); the fourth mouse became mildly diabetic (levels between 180 and 280 mg/dl) 3–4 weeks after supplementation. The histologic appearance of the islets from repleted mice resembled that of the streptozotocin-treated control mice.

Effect of Essential Fatty Acid Deficiency on Islet Resident Ia-Positive Cells. Because prior studies have shown a decrease in resident macrophages with essential fatty acid deficiency and have implicated this decrease in the anti-inflammatory effects of the deficiency state (20), we examined the effects of essential fatty acid deficiency on the population of resident Ia-positive cells within the islet. The number of Ia-positive cells per islet did not differ in untreated essential fatty acid-deficient and in untreated control mice; means ± SEM were 8.6 ± 0.81 and 7.7 ± 0.66 Ia-positive cells per islet, respectively.

DISCUSSION

Essential fatty acid deficiency effectively prevented multiple low-dose streptozotocin-induced diabetes in CD-1 mice. The incidence of diabetes was markedly reduced as was the severity of hyperglycemia when it occurred. Essential fatty acid deficiency also ameliorated the lymphocytic insulitis

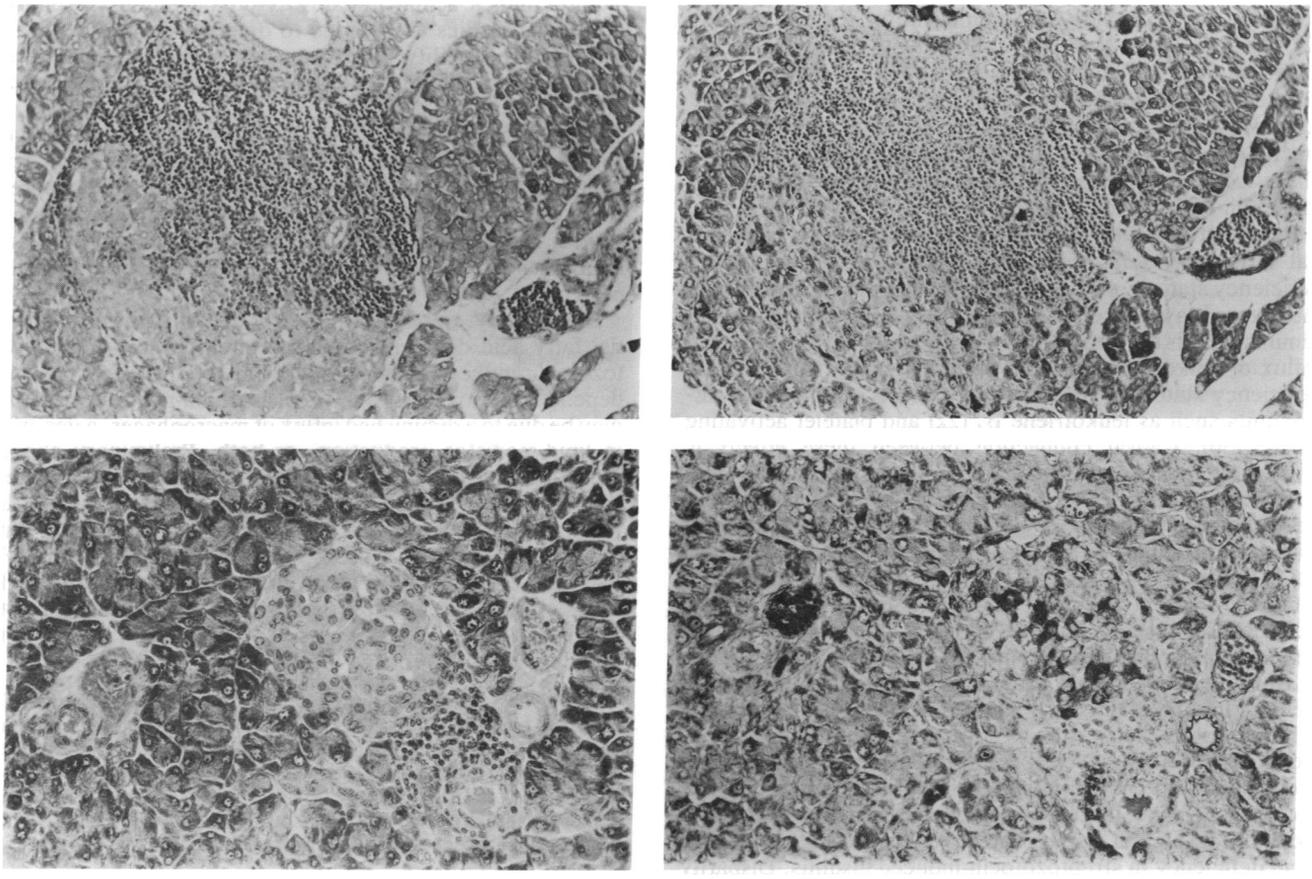

FIG. 2. (*Upper*) Histologic appearance of severely involved islets in low-dose streptozotocin-treated control mice (×190). There is extensive lymphocytic infiltration, destruction on normal islet architecture, and massive beta cell necrosis (*Left*). Note the almost complete absence of insulin-positive beta cells (*Right*). (*Lower*) Histologic appearance of an islet from a low-dose streptozotocin-treated essential fatty acid-deficient mouse (×300). Also note the scant lymphocytic peri-insulitis (*Left*) and the preservation of beta cell granularity (*Right*). (Plates on left are stained with hematoxylin/eosin; plates on right are stained with aldehyde fuschin.)

associated with injections of multiple low doses of streptozotocin leading to marked preservation of beta cells. Studies on the linoleate-repleted streptozotocin-treated essential fatty acid-deficient mice further suggest that essential fatty acid deficiency did not prevent the streptozotocin-induced beta cell injury because repleted animals developed diabetes and insulitis comparable to the controls. Our findings suggest that essential fatty acid deficiency prevents the full expression of the inflammatory response directed at the streptozotocin-altered beta cells.

Essential fatty acid deficiency did not prevent the inflammatory response entirely. In the streptozotocin-treated essential fatty acid-deficient mice, a peri-insulitis, usually involving one to several islets per histologic section, was present. Pancreata from streptozotocin-treated control mice had insulitis characterized by more extensive lymphocytic infiltrates, involvement of larger numbers of islets, insular invasion, and beta cell destruction. Why the peri-insular lymphocytes did not invade and destroy the islets in the essential fatty acid-deficient mice is not known. Lymphocytic infiltrates in mice with low-dose streptozotocin-induced insulitis have been previously characterized as being predominantly of the $L3T4^+$ and the $Lyt\text{-}2^+$ phenotypes (12). Possibly, the phenotype(s) of the peri-insular infiltrates is different from that in the controls or, perhaps, there is some defect in the activation of the cytotoxic T cells.

The role of the macrophage in antigen presentation and in the initiation of immune responses is well-known (for review, see ref. 28). Several recent studies have implicated macrophages as important in the evolution of type 1 diabetes in man, the spontaneously diabetic BB/Wistar rat, and the low-dose streptozotocin-treated mouse (for review, see ref. 12). Kolb and coworkers have shown with electron microscopy and with immunocytochemistry that macrophages infiltrate the islets before the lymphocytic insulitis in low-dose streptozotocin-induced diabetes (11, 12). The importance of macrophages is suggested by the fact that silica, a macrophage toxin, can almost completely prevent low-dose streptozotocin-induced diabetes (13).

Recent studies into the antiinflammatory effects of essential fatty acid deficiency suggest that the effects of the deficiency state on macrophages and their production of lipid mediators may be key. Essential fatty acid deficiency can diminish levels of resident macrophages (20, 21) as well as the influx of leukocytes into foci of inflammation (21, 29). The deficiency state also inhibits the synthesis of lipid chemoattractants such as leukotriene B_4 (22) and platelet activating factor (30). Causal connection between these effects is suggested by data showing that both essential fatty acid deficiency and BW 755C, an inhibitor of cyclooxygenase and lipoxygenase, inhibit *in vivo* generation of leukotriene B_4 and diminish the leukocyte response to an inflammatory stimulus (21). Essential fatty acid deficiency, however, apparently causes no defect in leukocyte chemotaxis: Essential fatty acid-deficient macrophages respond to chemotactic stimuli the same as controls (G.S. and J.B.L., unpublished data).

Because islets contain a population of resident Ia-positive cells as in the kidney (for review, see ref. 31), we examined whether islets isolated from untreated essential fatty acid-deficient mice had fewer Ia-positive cells than islets isolated from untreated control mice. No significant difference was found, suggesting that basal depletion of resident Ia-positive cells is not integral to the protective effect of essential fatty acid deficiency in streptozotocin-induced insulitis. Disparity in the behavior of islet and renal Ia-positive cells with essential fatty acid deficiency may be due to the different cell lineages. Glomerular Ia-positive cells appear to be macrophages as they stain with antimacrophage antibodies and are phagocytic. The nature of islet Ia-positive cells is uncertain; these cells could be dendritic cells rather than resident macrophages.

Because macrophages are the first cells to infiltrate the islets after injection of low-dose streptozotocin (11, 12), the rate of migration of Ia-positive macrophages into islets immediately after injection of low-dose streptozotocin could differ between the two groups and thus explain the protective effect of essential fatty acid deficiency or, as mentioned above, the diminished influx of macrophages into inflammatory foci with essential fatty acid deficiency could be due to decreases in lipid-mediator production (21). Preliminary results suggest that arachidonate metabolites play a role in the inflammatory response in this model of insulitis. Kolb-Bachofer and coworkers recently reported that BW 755C exerts a protective effect on low-dose streptozotocin-induced diabetes (14).

The results of our study and of studies from several other laboratories suggest that induction of diabetes with multiple low doses of streptozotocin requires at least three distinct steps: (*i*) induction of streptozotocin-induced neoantigens, (*ii*) influx of activated macrophages into the damaged islet, and (*iii*) lymphocytic activation and infiltration. Our repletion study demonstrates that induction of streptozotocin-induced neoantigens occurs in essential fatty acid deficiency. Because linoleate repletion was begun 3 days after the last dose of streptozotocin and because streptozotocin has a half-life of only 15 min, streptozotocin and its active metabolite, the methyl carbonium ion, would have been metabolized and excreted several days before supplementation was begun. This implies that the streptozotocin-modified beta cell continues to be immunogenic for at least some time and only requires linoleic acid to initiate a cytotoxic immune response. Additional studies are needed to determine whether a window of susceptibility exists or whether the streptozotocin-modified beta cell will be destroyed whenever supplementation occurs.

Note that the degree of protection from low-dose streptozotocin-induced diabetes offered by essential fatty acid deficiency was greater than that offered by immunosuppressive regimens directed primarily at lymphocytes. For instance, antilymphocytic serum alone (8), radiation (9), and antibodies to $L3T4^+$ lymphocytes (12) were only partially protective.

In summary, our findings clearly demonstrate that essential fatty acid deficiency prevents multiple low-dose streptozotocin-induced diabetes in CD-1 mice. This effect does not depend on prior depletion of resident Ia-positive cells but may be due to a diminished influx of macrophages, a decrease in lipid-mediator production, or both. Preliminary experiments to determine the generality of our observation suggest that essential fatty acid deficiency will also prevent the spontaneous diabetes and thyroiditis in BB/Wistar rats (G.S., A. Rossini, J. Mordes, E. Handler, P.E.L., J.R.W., and J.B.L., unpublished data). Further study into the mechanisms underlying our observations may provide insights into the pathogenesis of type 1 diabetes mellitus and strategies to ameliorate or prevent the insulitis.

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