Fetal or Neonatal Low-Glycotoxin Environment Prevents Autoimmune Diabetes in NOD Mice

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Advanced glycation end products (AGEs) are implicated in β-cell oxidant stress. Diet-derived AGE (dAGE) are shown to contribute to end-organ toxicity attributed to diabetes. To assess the role of dAGE on type 1 diabetes, NOD mice were exposed to a high-AGE diet (H-AGE) and to a nutritionally similar diet with approximate fivefold-lower levels of N^{ϵ}-carboxymethyllysine (CML) and methylglyoxal-derivatives (MG) (L-AGE). Suppression of serum CML and MG in L-AGE–fed mice was marked by suppression of diabetes (H-AGE mice >94% vs. L-AGE mice 33% in founder $[F]_0$, 14% in F_1, and 13% in F_2 offspring, $P < 0.006$) and by a delay in disease onset (4-month lag). Survival for L-AGE mice was 76 vs. 0% after 44 weeks of H-AGE mice. Reduced insulitis in L-AGE versus H-AGE mice ($P < 0.01$) was marked by GAD- and insulin-unresponsive pancreatic interleukin (IL)-4–positive CD4+ cells compared with the GAD- and insulin-responsive interferon (IFN)-γ–positive T-cells from H-AGE mice ($P < 0.005$). Splenocytes from L-AGE mice consisted of GAD- and insulin-responsive IL-10–positive CD4+ cells compared with the IFN-γ–positive T-cells from H-AGE mice ($P < 0.005$). Therefore, high AGE intake may provide excess antigenic stimulus for T-cell–mediated diabetes or direct β-cell injury in NOD mice; both processes are ameliorated by maternal or neonatal exposure to L-AGE nutrition. *Diabetes* 52:1441–1448, 2003

Insulin-dependent diabetes (type 1 diabetes) is an autoimmune disease resulting from T-cell–mediated destruction of pancreatic islet β-cells (1). However, the initial events of this process are incompletely understood. While both MHC and non-MHC genes have been implicated in the islet autoimmunity of both mice and humans (2,3), T-cell autoantigens, such as GAD or insulin, as well as environmental factors remain as strong candidates (2,4–7). The interplay between heritable aberrations, and environmental influences can lead to disruption of immune tolerance, T-cell activation, and β-cell injury (2,3,6,7). Diet is a strong environmental determinant of type 1 diabetes (8–14), but the underlying mechanisms involved are not fully elucidated (10).

The reactive, dicarbonyl derivatives of glucose-protein or glucose-lipid interactions, termed advanced glycation end products (AGEs), such as N^{ϵ}-carboxymethyllysine (CML) and methylglyoxal (MG), are implicated in a wide range of diseases related to diabetes and aging (15,16), including β-cell injury and apoptosis, via reactive oxygen species (ROS)-dependent processes (17,18). AGE receptors found on immune cells mediate cell activation and cytokine expression, and in T-cells, IFN-γ secretion (19–21).

Diet constitutes an important exogenous source of highly reactive AGE (22–24). A direct correlation has been shown between ingested AGE and that found in circulation (25–27). Excess oral AGE intake results in an imbalance of AGE homeostasis, leading to significant diabetes-like pathology (25–33). On the contrary, β-cell dysfunction and diabetic complications are suppressed by AGE inhibitors (34,26) or by restriction of dietary AGE (25–33). In addition, a significant reduction of circulating AGE levels was observed in renal failure patients under peritoneal dialysis treatment after 4 weeks on a low–AGE content diet (35). Recently, food-derived AGE, rich in MG and CML derivatives, among others, were found to be potent inducers of oxidative stress and inflammatory cell activation in a manner reversible by antioxidants and anti-AGE agents, such as aminoguanidine (36). Furthermore, in type 2 diabetic *db/db* mice, dietary AGE restriction suppressed β-cell damage, thereby maintaining normal glucose homeostasis (31).

Based on the above, we investigated whether early exposure to AGE taken in with regular nutrients has a role in the pathogenesis of type 1 diabetes in NOD mice, a model of human diabetes (3,5).

RESEARCH DESIGN AND METHODS

Mouse dietary formulas. CML-BSA and methylglyoxal-BSA derivatives (MG) in rodent food were assessed by enzyme-linked immunosorbent assay (ELISA) using monoclonal antibodies anti-CML-KLH (4G9) (Alteon, Northvale, NJ) (ELISA-1) or anti-MG-ovalbumin (MG3D11) (ELISA-2), as described (36,37).

We used semipurified standard diet, AIN-93G (Bio-Serv, Frenchtown, NJ), which is normally exposed to heat (at 100°C × 20–60 s and at 125°C × 20–30 min), containing 535 CML-like AGE units/mg and 18 nmol/mg MG-derivatives/mg, herein termed "high-AGE diet" (H-AGE). The identical chow mix, prepared without the second step of heating, contained fivefold less CML-like AGE and 4.5-fold less MG-derived epitopes, as compared with H-AGE chow, and is thus termed "low-AGE" (L-AGE) formula (Table 1).

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Received for publication 20 August 2001 and accepted in revised form 10 February 2003

CML, N^{ϵ}-carboxymethyllysine; dAGE, diet-derived AGE; ELISA, enzyme-linked immunosorbent assay; GALT, gut-associated lymphoid tissue; H&E, hematoxylin and eosin; H-AGE, high-AGE diet; IFN, interferon; IGTT, intraperitoneal glucose tolerance test; IL, interleukin; L-AGE, low-AGE diet; MG, methylglyoxal; PI, proinsulin; pLy, pancreatic lymphocytes; ROS, reactive oxygen species; sAGE, serum AGE; SI, stimulation index; sLy, splenic lymphocytes; UA, urinary albumin.

TABLE 1
Characteristics of diet formulas

	AIN recommendations*	AIN-93G†	
		H-AGE	L-AGE
Nutrients (%)			
Protein	20	18.4	
Fat	5	7.2	
Carbohydrate	65	58.6	
Total calories (kcal/g)	4	3.9	
AGE content			
CML (units/mg)‡		535	107
MG (nmol/mg)¶		18	4

*See ref. 38. †Ingredients: casein, cornstarch, dyetrose, sucrose, cellulose, soybean oil, t-butylhydroquinone, salt mix, vitamin mix, L-cystine, choline bitartrate; ‡CML determined by 4G9 mab (37). ¶MG determined by MG-3D11 mab (36).

Macronutrients (protein 18.4%, carbohydrate 58.6%, fat 7.2%) were identical in these diets, and micronutrients were mostly in excess of the daily requirements, following standard processing (38). Both formulas were pelleted by the manufacturer and kept at 4°C.

Mice. Because female NOD mice have a much higher rate of diabetes than the male NOD mice, only female mice were studied. Prediabetic NOD founder (F_0) mice (34 females and 12 brother-sister mating pairs aged 6 weeks), obtained from Dr. M. Hattori (Joslin Diabetes Center, Boston, MA), were housed in a pathogen-free environment. Upon arrival, F_0 mice as well as the first (F_1, n = 55) and second (F_2, n = 51) generation offspring were housed at the Center for Laboratory Animal Science, Mount Sinai School of Medicine. Animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee.

Baseline body weights and blood and urine samples were obtained, and mice were randomly assigned to either H-AGE or L-AGE diet and followed for up to 56 weeks of age. Of the F_0 mice, 22 females and 8 mating pairs were placed on L-AGE diet, and 12 females and 4 mating pairs were placed on the H-AGE diet, immediately upon arrival (6 weeks of age). After weaning (3 weeks), the F_1 (n = 55) and F_2 (n = 51) generations' offspring were assigned to diets: 21 of F_1 and 30 of F_2 female NOD mice continued on the L-AGE diet, while 18 of F_1 and 21 of F_2 female NOD mice continued on the H-AGE diet. An additional 16 females of F_1 offspring from L-AGE–fed mothers were switched to the H-AGE diet, after weaning.

Food and water intake were measured daily for 1 week and weekly thereafter. Body weight and urinary glucose levels (Dextrostix; Bayer, Indianapolis, IN) were checked once per week until onset of glucosuria and by tail vein blood thereafter (Glucometer Elite; Bayer, Mishawaka, IN). Overt diabetes was defined as persistent hyperglycemia (>250 mg/dl) for over a week. Diabetic mice were treated with insulin (1–3 units of combined regular-NPH insulin, 1:1) twice daily to maintain blood glucose levels at 150–200 mg/dl for up to 56 weeks.

Twelve-hour fasting blood samples (by retroorbital puncture) and 24-h urine samples were collected at 1- to 2-month intervals and stored at −80°C. Subgroups from F_1 on either diet were sacrificed at 9, 12, and 16 weeks for collection of blood and tissue specimens.

Metabolic studies. At 16 weeks of age, an intraperitoneal glucose tolerance test (IGTT, 5% dextrose solution; 2 mg/g body wt) was performed in groups of 12 mice from four different litters after an overnight fast (13–15 h). Blood glucose (Elite Glucometer; Bayer) and insulin levels were measured before and at half-hour intervals up to 120 min. Insulin levels were measured by ELISA (Ultra-Sensitive Mouse Insulin Kit; Alpco Diagnostics, Windham, NH).

Serum and urine AGE levels were determined by ELISA-1, as described (36,37). Urinary albumin (UA) levels were measured with an anti-mouse albumin–based ELISA kit (Bethyl Laboratories, Montgomery, TX); urinary creatinine was assessed by a standard colorimetric method (Stanbio Laboratory, San Antonio, TX). Renal function was expressed as the albumin-to-creatinine ratio (28).

Immunohistology and flow cytometry. After asphyxiation by exposure to CO_2, pancreatic tissues were fixed in 10% formaldehyde, and 4-μm paraffin sections were stained with hematoxylin and eosin (H&E). A staff pathologist analyzed 15–20 islets/pancreas in a blinded fashion, using a grading system, in which 0 = no evidence of infiltration, I = peri-islet infiltration, II = 25%, III = >25–45%, IV = >50% infiltration of each islet, and V = complete loss or only remnants of islets seen (39).

For flow cytometry, pancreatic and spleen tissues from 16-week-old mice (n = 8/group) were processed for lymphocyte isolation (40). Suspensions of pancreatic lymphocytes (pLy) were obtained after gentle mincing with fine scissors and digesting with collagenase type V (4 mg/ml) and DNase type II (100 units/ml) (Sigma Chemicals, St. Louis, MO) in RPMI-1640 for 15 min at 37°C. After centrifugation, single-cell suspensions were obtained by continuous pipetting in a solution containing trypsin/EDTA (1 ml/ml) (Gibco/BRL) and DNase type II (2,000 units/ml) at 37°C for 10 min. Single-cell suspensions of splenic lymphocytes (sLy) were obtained by gently pressing the spleens through wire mesh screens and rinsing with RPMI-1640 supplemented with 10% FBS and 2% penicillin/streptomycin (Gibco/BRL, Gaithersburg, MD). Red cells were lysed by 0.84% ammonium chloride.

For labeling pancreatic lymphocytes, anti-CD4FITC, anti-IL-4 PE (Pharmingen, San Diego, CA), and anti–IFN-γ antibodies (Pharminogen) were used. After a 6-h incubation at 37°C in the presence of monensin (0.1 μg/ml, Sigma) to block cytokine secretion, cells were stained with the relevant anti-CD4 antibody at 4°C for 30 min, permeabilized with saponin 0.1%, and stained with each antibody or isotype control. Cells (5×10^3/sample) gated on a window encompassing the sLy and pLy population were analyzed by flow cytometry (EPICS Profile II Analyzer; Coulter, Hialeah, FL) (40).

Proliferation and lymphokine expression assays. pLy (5×10^5/well) from 16-week-old F_1 mice (n = 6/group) were incubated for 72 h at 37°C with antigen-presenting cells (APCs) (6×10^5/well) (sLy from the H-AGE fed mice, treated with mitomycin [1 mg/ml; Sigma] and incubated at 37°C for 20 min) in the presence or absence of ConA, the immunodominant peptide of GAD65 217-36 and mouse proinsulin (PI 9-23) (10 μg/ml), both of which were obtained from the Peptide Synthesis Core (Department of Molecular Biology, Mount Sinai School of Medicine). Proliferative responses for pLy were confirmed and expressed as stimulation index (SI) (calculated as cpm in the presence of stimulant/cpm without stimulant).

In addition, sLy (1×10^6/well) was incubated for 72 h at 37°C in the presence or absence of ConA (10 μg/ml), GAD65 (10 μg/ml), or PI (10 μg/ml). Proliferative response was determined 72 h after incubation with ^{3}H-TdR. sLy from 16-week-old mice (n = 12/group) were also incubated with various doses of anti-CD3 (Pharmingen), and proliferative response was measured 48 h after incubation with ^{3}H-TdR. ConA was used in all T-cell experiments as a nonspecific stimulant to estimate proliferative response. To evaluate sLy gene expression for IFN-γ, IL-4, and IL-10, total RNA isolated from the spleens of 12-week-old female NOD mice (n = 5/group) was reverse transcribed to cDNA, and RT-PCR was performed as described. β-Actin was amplified at the same time for each sample, and the data were expressed as the ratio of cytokine to β-actin mRNA (41).

Statistical analysis. All values are expressed as means ± SE. Two-tailed unpaired t test (Mann-Whitney) or Student's t test was used as needed to evaluate differences between means of grouped data from mice fed with H-AGE and L-AGE diets. Differences were considered significant if P was <0.05.

RESULTS

Dietary AGE content and serum AGE levels. Food and water intake were similar among age- and diabetes-matched groups during the study period. There were no significant differences in body weights between H-AGE and L-AGE diet groups. The only weight loss was that preceding death in type 1 diabetic mice (Table 2).

Based on equivalent food intake, H-AGE fed mice ingested approximately fivefold more AGE than the L-AGE–fed mice. This was clearly reflected in the fasting serum AGE (sAGE) of both diabetic and nondiabetic groups throughout the study (Fig. 1*A* and *B*). Baseline sAGE levels in NOD mice decline in a time-dependent manner in both diabetic and nondiabetic groups fed the L-AGE diet ($P <$ 0.025) (Fig. 1*A* and *B*). Greater AGE ingestion by the H-AGE–fed mice was associated with greater urinary AGE excretion compared with L-AGE–fed mice in both the diabetic and nondiabetic groups ($P < 0.002$) (Table 2).

Dietary AGE and diabetes incidence and survival. A striking reduction in the cumulative incidence of type 1 diabetes soon became evident in the AGE-restricted

TABLE 2
Characteristics of NOD mice exposed to H-AGE and L-AGE diets

	H-AGE diet		L-AGE diet	
	Baseline	End	Baseline	End
Body weight (g)	15.2 ± 1	23.5 ± 1.2	14.5 ± 1	25 ± 2.2
Fasting blood glucose (mg/dl)	55 ± 18	195 ± 48	60 ± 11	205 ± 61
Serum creatinine (mg/dl)	0.61 ± 0.2	0.68 ± 0.1	0.62 ± 0.2	0.60 ± 0.1
Albumin/creatinine excretion (mg/mg)	1.5 ± 0.1	8.3 ± 0.5†	1.2 ± 0.1	1.7 ± 0.2
AGE/creatinine excretion (units/mg)	1.4 ± 0.1	3.8 ± 0.9‡	1.7 ± 0.1	2.1 ± 0.3

Data are means ± SD. *Refers to AIN-93G, as defined in Table 1 (ref. 36); †$P < 0.002$; ‡$P < 0.025$.

groups, unlike those placed on regular (H-AGE) diet. Of 16 F_0 H-AGE–fed mice, 15 (94%) developed diabetes by 25 weeks of age, whereas only 6 of 30 L-AGE–fed mice (33%) became diabetic ($P = 0.000$) (Fig. 2*A*) or with a delay of 15 weeks (40 weeks of age) (Fig. 2*D*). From the F_1 group ($n = 55$), 11 of 18 H-AGE–fed mice (61%) were diabetic by 25 weeks, as compared with only 3 of 21 (14%) of L-AGE mice developing diabetes by ~40 weeks ($P < 0.006$) (Fig. 2*B*) or with a 15-week lag (Fig. 2*E*). Similar results were obtained with the F_2 offspring: 13 of 21 (62%) H-AGE–fed mice were diabetic by 25 weeks, as compared with 4 of 30 (13.3%) L-AGE–fed mice by 40 weeks ($P = 0.000$) (Fig. 2*C*) (Fig. 2*F*). A large proportion of a subgroup of F_1 H-AGE mice, derived from L-age–fed mothers (9 of 16, or 56%) became diabetic by 24 weeks (no lag) (Fig. 2*B* and *E*). At the point of maximal incidence of diabetes for H-AGE mice (~50% at ~25 weeks), only <5% of the L-AGE mice were affected (Fig. 2*D*–*F*), with a marked shift to the right (~40 weeks). (Fig. 2*D*–*F*).

Overall, 76% of the founder mice fed the L-AGE diet were alive up to 56 weeks, whereas none of the H-AGE fed mice survived after 44 weeks, including diabetic mice treated with insulin (L-AGE vs. H-AGE, $P < 0.0001$).

Dietary AGE and β-cell function. At ~16 weeks of age, prediabetic L-AGE F_1 mice exhibited significantly lower fasting blood glucose and higher plasma insulin levels compared with age-matched H-AGE F_1 mice ($P < 0.021$ and $P < 0.009$, respectively) (Fig. 3*A* and *B*). Also, a significantly lower glycemic response to IGTT (Fig. 3*A*), with greater insulin responses to glucose, were observed in the L-AGE mice compared with H-AGE mice (Fig. 3*B*).

Histological examination of pancreas. Pancreata of 9-week-old prediabetic mice ($n = 8$/group, F_1) and 40-week-old diabetic ($n = 10$, F_0) and nondiabetic ($n = 15$, F_0) mice from either diet were evaluated. At 9 weeks, islets H-AGE–fed from mice revealed severe inflammatory infiltration, essentially obliterating the normal islet architecture (>50% of the islet surface affected, grades III–V) (39). In contrast, islets from the L-AGE–fed group showed only occasional islets with lymphocytic infiltration (<24% of each islet, grade <II) (Fig. 4*A* and *B*). Quantitative analysis revealed an insulitis score of 4.1 ± 1.3 for the H-AGE–fed group versus 1.2 ± 0.5 for the L-AGE–fed group ($P < 0.01$).

At 48 weeks, compared with the islets from diabetic H-AGE mice (100% grade >III or >50% of each islet), only 20% of islets from the diabetic L-AGE mice were affected as seriously, the remaining 80% exhibiting mild insulitis, grades I–II. Of the nondiabetic H-AGE–fed mice, 50% exhibited severe insulitis (>grade III), as compared with only 10% of L-AGE with same degree of damage and the rest showing only mild or no insulitis (grades I and II).

Pancreatic lymphocyte populations. The total number of pLy was markedly suppressed in 16-week-old L-AGE–fed mice, as compared with the H-AGE–fed mice (L-AGE $0.10 \pm 0.15 \times 10^7$ vs. H-AGE $7.7 \pm 5.6 \times 10^7$, $P < 0.001$, respectively) (Fig. 5*A*). Also, CD4+ pLy were approximately threefold lower in the L-AGE compared with H-AGE fed mice (L-AGE 8.3 ± 4.4% vs. H-AGE 23.4 ± 6.1%, $P = 0.000$) (Fig. 5*B*). A fivefold greater percentage of CD4+ pLy was positive for IL-4 in the L-AGE group, whereas the reverse was observed in the H-AGE–fed group (L-AGE 25.9 ± 3.22% vs. H-AGE 5.52 ± 1.15%, $P < 0.009$) (Fig. 5*C*). In contrast, a threefold greater percentage of CD4+ from the H-AGE group were IFN-γ positive, as

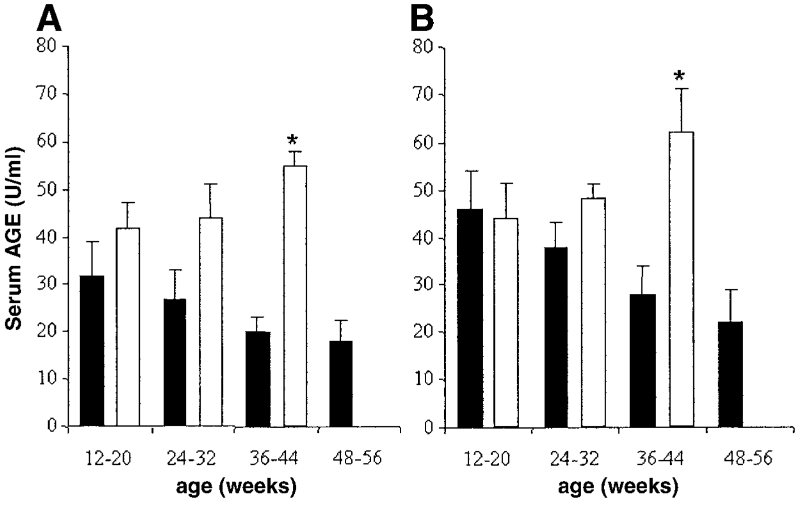

FIG. 1. Serum AGE levels in diabetic and nondiabetic female NOD mice exposed to L-AGE or H-AGE diet. After exposure to either regular AIN-93G (H-AGE) or L-AGE diet, fasting serum samples were obtained for AGE testing at various time points, using a competitive CML-sensitive ELISA (4G9 mab), as described. (*A*): Nondiabetic (*A*) and diabetic (*B*) NOD mice on H-AGE (□) and L-AGE (■). Data are AGE units/ml and the mean ± SE of three independent tests, each performed in triplicate. *$P <$ 0.025.

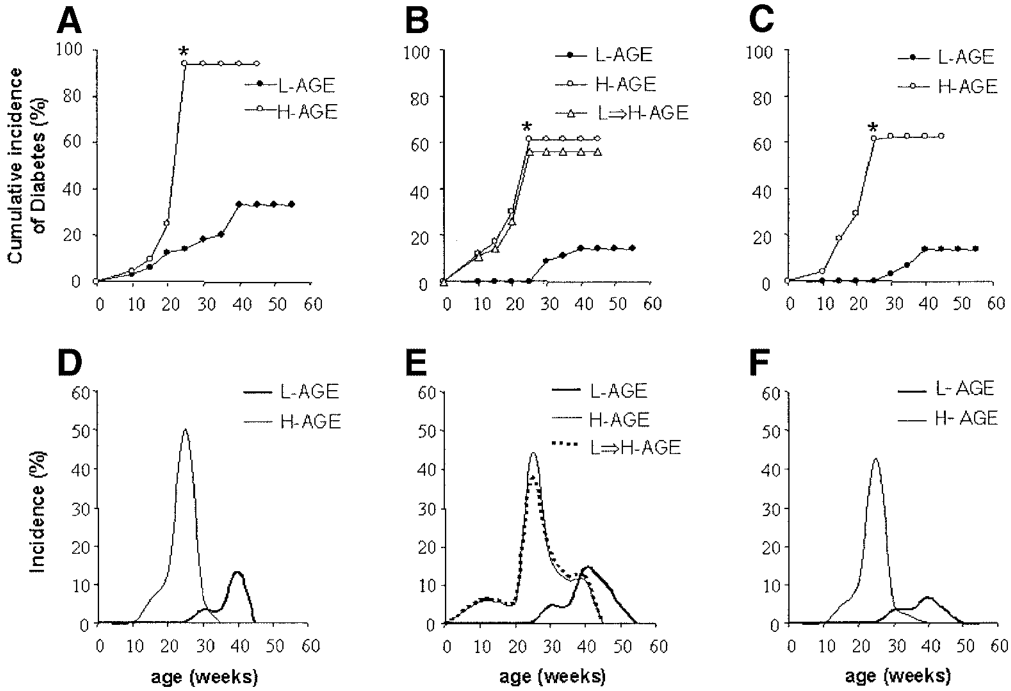

FIG. 2. Incidence of type 1 diabetes in F_0, F_1, and F_2 generations of female NOD mice exposed to L-AGE or H-AGE diet. *A*: By 6 weeks of age, F_0 mice were placed on H-AGE (n = 16) or L-AGE diet (n = 30). *B*: After weaning (3 weeks), F_1 offspring were placed on H-AGE (n = 18) or L-AGE diet (n = 21). Also, a subgroup of F_1 mice (n = 16) from L-age–fed mothers was switched to H-AGE diet (L- to H-AGE). *C*: After weaning (3 weeks), F_2 offspring were placed on an H-AGE (n = 21) or L-AGE diet (n = 30). *A–C*: Cumulative incidence of type 1 diabetes. *D–F*: Incidence of type 1 diabetes in F_0, F_1, and F_2 mice on H-AGE diet (○) on H-AGE diet from L-AGE mothers (L- to H-AGE) (△) and L-AGE diet (●). Diabetes incidence was monitored for 56 weeks. *P = 0.000 (*A*), *P = 0.006 (*B*), *P = 0.000 (*C*) for H-AGE or L→H-AGE (L- to H-AGE diet) vs. L-AGE groups.

compared with those from L-AGE mice (H-AGE 19.3 ± 1.15 vs. L-AGE 6.67 ± 1.84%, $P < 0.009$) (Fig. 5*C*).

The splenic T-cell CD4+-to-CD8+ ratio (3.5:1) was not different between the diet groups. Thus, sLy isolated from 12-week-old mice were tested for lymphokine mRNA expression: L-AGE mice exhibited low levels of IFN-γ and low IL-4 but a high IL-10–to–β-actin mRNA ratio (IFN-γ 0.04 ± 0.008, IL-4 0.07 ± 0.002, IL-10 2.98 ± 0.6), as compared with sLy from H-AGE–fed mice (IFN-γ 1.75 ± 0.5, IL-4 0.22 ± 0.06, IL-10 0.38 ± 0.04, $P < 0.001$, respectively) (Fig. 6*A*).

Proliferation of pLy and sLy. The pLy from H-AGE–fed mice showed a significantly greater proliferative response to GAD (10 μg/ml) and to PI peptides (10 μg/ml) compared with that of pLy from L-AGE mice ($P < 0.011$ and $P < 0.005$, respectively) (Fig. 5*D*).

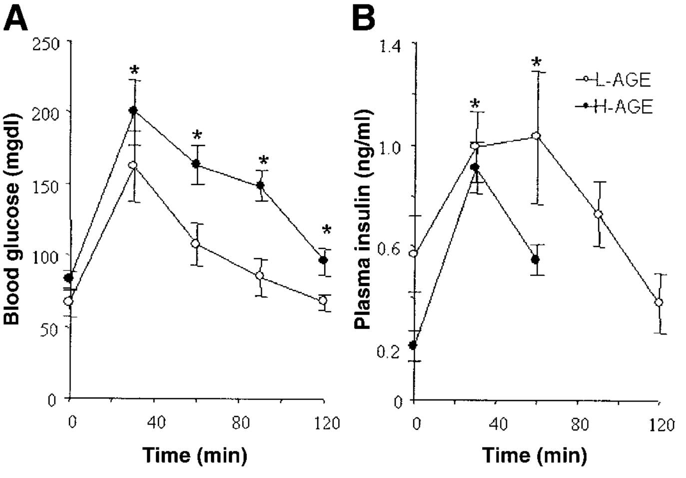

FIG. 3. β-Cell function of NOD mice exposed to L-AGE or H-AGE diet. After glucose challenge (IGTT, 5% dextrose solution, 2 mg/g body wt i.p.) in 16-week-old nondiabetic female F_1 mice from H-AGE–fed (●) and L-AGE–fed groups (○) (n = 12/group). Glucose (*A*) and insulin (*B*) responses were estimated at half-hour intervals up to 120 min. Data are the mean ± SE of 12 measurements per time point per group. *A*: *Glucose in H-AGE vs. L-AGE at 0 min, P = 0.021; at 30 min, P = 0.036; at 60 min, P = 0.009; at 90 min, P = 0.009; and at 120 min, P = 0.009. *B*: *Insulin in H-AGE vs. L-AGE at 0 min, P = 0.009; at 30 min, P = 0.009; and at 60 min, P = 0.009.

By comparison, L-AGE mouse–derived sLy proliferated more rigorously than sLy from H-AGE mice in response to GAD (10 μg/ml) and to PI (10 μg/ml) ($P < 0.02$, $P < 0.017$, respectively) (Fig. 6*B*), as they did in response to anti-CD3 ($P < 0.005$) (Fig. 6*C*).

DISCUSSION

The current study demonstrates that restricted exposure to diet-derived glycotoxins leads to marked and sustained blockade of type 1 diabetes in the genetically susceptible NOD mouse. The earlier in life the restriction was applied, the greater the decrease in incidence of disease. These effects were marked by suppression of islet infiltration by β-cell cytotoxic T-cells and islet toxicity. In addition, significantly delayed onset, reduced severity of disease, and marked increase in overall survival occurred.

While these effects were readily attributable to the food, they were not due to differences in nutrient composition, as the diets used were of a single origin, with equivalent energy profiles (38). A prominent feature, however, was their distinctly different glycotoxin content. A large proportion of CML or MG derivatives found in the regular or H-AGE diet did not develop in the L-AGE preparation, which resulted from the shorter heat exposure during processing. Indeed, methods of food processing (heating, sterilizing, and ionizing) impact on diverse unstable α-β-dicarbonyl derivatives of glyco- and lipoxidation reactions, while temperature and time are among the principal rate-determinants for these cascades (22–24). A significant proportion of AGE (~10%) is absorbed orally, including—though not exclusively—tissue-toxic species (25–27). AGE are found capable of β-cell damage (17,18,31,34), as well as of altered immune cell activation (19–21). Thus, the

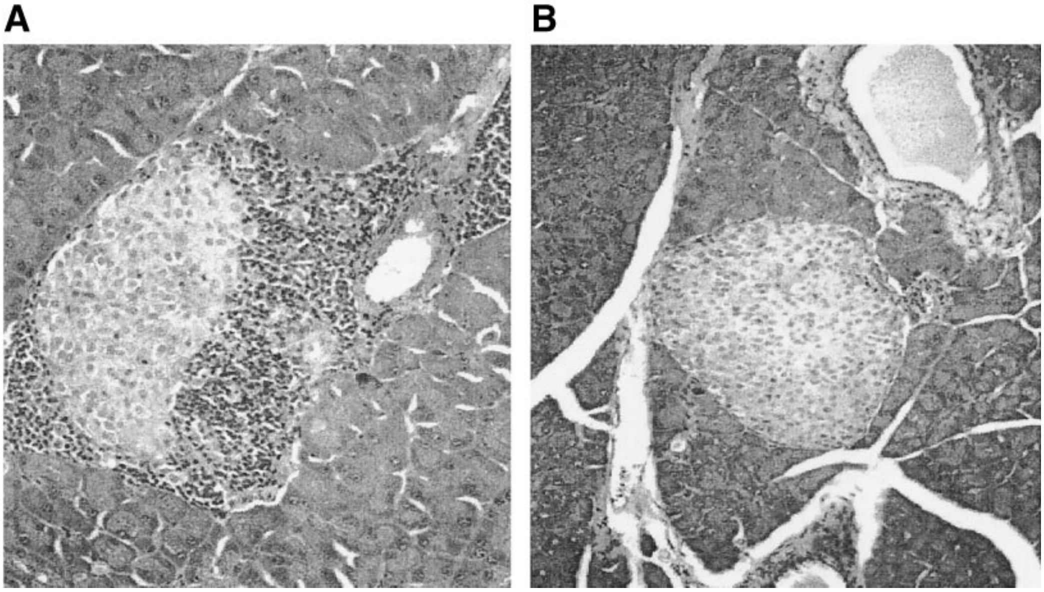

FIG. 4. Islet morphology in nondiabetic F_1 NOD mice exposed to L-AGE or H-AGE diet. *A*: Pancreatic tissues retrieved from 9-week-old F_1 H-AGE–fed mice, stained by H&E, showed severe mononuclear cell infiltration and disruption of pancreatic islet architecture. *B*: Islets of age-matched F_1 NOD mice from the L-AGE–fed group did not exhibit these changes. Magnification ×400.

possibility that food-derived AGE contribute to autoimmune diabetes was raised, herein viewed from the perspective of the diabetes-protective effect of a diet low in AGE applied to diabetes-prone NOD mice.

Diabetes suppressive effects under L-AGE feeding were significant in founder mice, initiated at 3 or 6 weeks of age, and were extended throughout two generations of offspring, F_1 and F_2, if kept on the L-AGE maternal regimen, yielding a diabetes-free rate of ~86% and greatly increased survival rates (by ~76%) for up to 56 weeks of age. As with other diabetogenic factors (8–14), type 1 diabetes reduction (~33 to 14%) coincided with initiation to the L-AGE diet during the perinatal period. Indeed, autoimmune cell infiltration of islets occurs soon after the 3-week weaning in NOD mice (42,43). The greatest disease prevention (<14%) was associated with exposure to a low in AGE maternal environment, suggesting that toxic AGE are transportable via the placenta. However, the protective maternal effect of the L-AGE diet was reversed after crossing over to the H-AGE diet, as readily as the normally H-AGE maternal effect was reversed by the L-AGE diet, when applied at weaning (3 or 6 weeks). These data confirmed the plasticity inherent in this period and reenforced the importance of "dose," "time," and "duration" of exposure to toxic factor(s) (12).

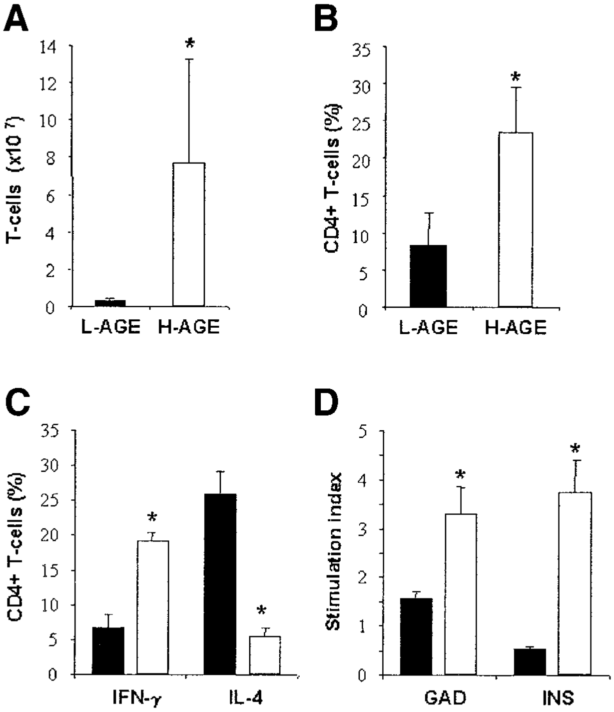

FIG. 5. Pancreatic T-cell responses in nondiabetic NOD mice exposed to L-AGE or H-AGE diet. *A*: Total infiltrating pancreatic T-cells isolated from pancreata of 16-week-old female NOD mice were measured in H-AGE- or L-AGE–fed mice (n = 8/group), *P < 0.001. *B*: The percentage of CD4+ pancreatic T-cells was assessed by anti-CD4+ fluoroscein isothiocyananate (FITC) using fluorescence-activated cell sorter (FACS) analysis, P = 0.000. *C*: The cytokine profile of CD4+ T-cells was evaluated by labeling with anti–IFN-γ or anti–IL-4 antibodies (FACS analysis), *P < 0.009, respectively. *D*: Proliferation of pancreatic T-cells (5 × 10^5/well), incubated for 72 h at 37°C with GAD and insulin (10 μg/ml), was estimated by ^{3}H-TdR (1 μCi/well), *P < 0.011 for GAD and <0.005 for insulin. Data are the means ± SE of eight measurements/group in H-AGE (□) and L-AGE (■) mice.

Thus, the majority of mice exposed to L-AGE environment maternally or at weaning displayed modest insulitis and no diabetes for >1 year. A milder insulitis and a milder diabetes characterized L-AGE mice that did become diabetic, as compared with those with severely damaged islets and overt diabetes seen in H-AGE–fed mice by ~25 weeks. Further evidence was obtained by assessing β-cell function: 16-week-old prediabetic L-AGE NOD mice exhibited near-normal glucose and insulin responses to glucose challenge compared with the typically dysfunctional pattern of age-matched H-AGE–fed mice.

The diabetogenic AGE components in the H-AGE preparation used herein may include CML or MG-derived compounds, although this remains to be confirmed. Also, coexisting non-AGE substances in that formula cannot be ruled out (8–14). However, a plausible link may be invoked between ordinary foods, such as casein in milk, which has been implicated in type 1 diabetes (44), and common glycotoxins; heating milk during pasteurization increases its AGE content (24) and its putative β-cell toxicity. Of note, unlike most other "diabetogenic" factors,

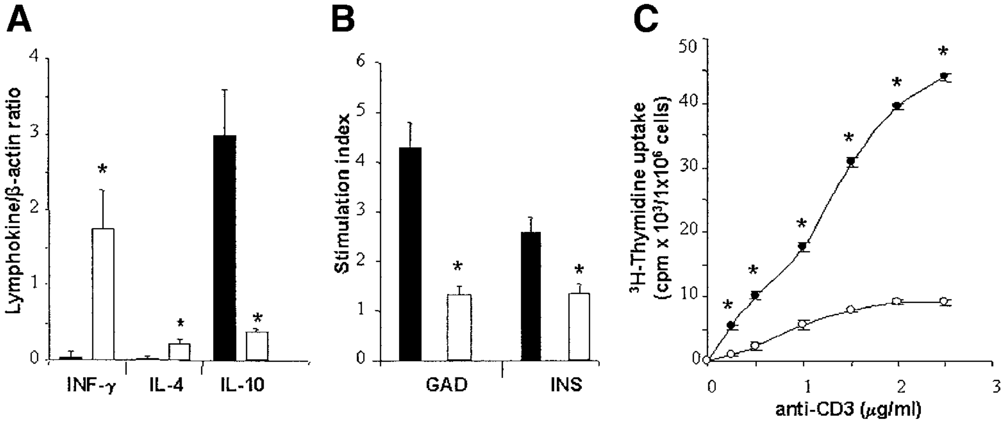

FIG. 6. Splenocyte responses in nondiabetic NOD mice exposed to L-AGE or H-AGE diet. *A*: Total RNA isolated from spleens of 12-week-old NOD mice (n = 5/group) was transcribed to cDNA, and RT-PCR was performed to detect IFN-γ, IL-4, and IL-10 mRNA levels, expressed as the mean ± SE cytokine–to–β-actin RNA ratio. *P values <0.001. *B*: Proliferation of spleen T-cells (1 × 10^6/well), incubated for 72 h at 37°C with GAD and PI (10 μg/ml), was estimated by ^{3}H-TdR (1 μCi/well), shown as SI (cpm in the presence of stimulant/cpm in the absence of stimulant). *P values of H-AGE vs. L-AGE groups for GAD <0.02; for insulin <0.017. *C*: Proliferation of splenocytes (1 × 10^6 cells/well), incubated for 72 h at 37°C with different doses of anti-CD3 (0.25–2.5 μg/ml), was estimated by ^{3}H-TdR. Data are means ± SE in H-AGE (○) and L-AGE (●) mice. *P refers to differences <0.005 between diet groups.

AGEs are known to contribute to other diabetic organ toxicity (15,25–33). Of interest, CML and MG-related derivatives were negligible in L-AGE diet, relative to the H-AGE diet; otherwise, these two preparations were identical (37). While no class of heat-enhanced compounds has been implicated in the etiology of type 1 diabetes thus far, AGEs are known to manifest an intriguing array of cellular effects, namely oxidant stress, nuclear factor (NF)-κB activation, or apoptosis and death (15,17,34,36), and may constitute such a class.

With regard to the targeted tissue, diabetogenic factors are thought to act through the immune system, principally via β-cell cytotoxic T-cells (1,2,6,7,10). The hypothesis introduced herein is no exception. A marked suppression of total pLy was observed in the L-AGE–fed mice (~>15-fold) compared with the H-AGE NOD group fed a regular diet. In type 1 diabetes in humans and NOD mice maintained on common diets, such as the H-AGE food, cytotoxic CD8+ and CD4+ cells, which are generated at the gut-associated lymphoid tissue (GALT) early in life, dominate islet infiltrates and disease inducement (45,46). However, in the present studies, islets from the L-AGE–fed mice displayed a pattern of predominantly IL-4–positive CD4+ cells. There were virtually no infiltrates in islets from two generations of mice exposed to L-AGE nutrients during the fetal stage or early in life. In these mice, there was also a marked unresponsiveness of pancreatic T-cells to β-cell antigens, insulin, and GAD. This might indicate blocked autoreactive T-cell recruitment due to low local expression of these antigens under a “safer” AGE-poor diet (47). The vigorous proliferative response to the same antigens by sLy from the L-AGE–fed mice, however, suggested the opposite. Of interest, sLy in these mice consisted largely of CD4+ IL-10–positive and IFN-γ–negative T-cells, suggesting that active and able Th2 T-cells were released systemically under L-AGE feeding. In fact, the rigorous sLy response to GAD and insulin pointed to preexisting memory to these self-antigens (47). It can be thus speculated that the L-AGE diet provided the young GALT system with a low dose of certain AGE-related antigen(s), which enabled Th2 cells to actively suppress nondeleted autoreactive T-cells (45–47). The opposite might be expected in an environment of excess AGE antigen(s), as provided by the H-AGE diet, whereby cytotoxic T-cells prevail.

Because normally dying β-cells liberate cellular components that stimulate autoreactive T-cells (7), the notable absence of such clones from L-AGE–derived islets was surprising. An alternate source and mode of presentation of such “self-antigens” might be postulated. For instance, animal- or plant-derived GAD peptides (48,49) could be modified by, and or absorbed together with, AGE epitopes derived from heat-processed food. The specific steps of such a mechanism are uncertain at this time. However, a rarely discussed but relevant property of glycotoxins is their receptor-dependent action on primed T-cells to produce IFN-γ (21). It is plausible that early exposure of GALT to excess diet-derived AGE-peptides (e.g., AGE-GAD peptides) results in a population of nondeleted autoreactive T-cells, which in a predisposed setting, as in the NOD mouse, offsets the balance in favor of tissue-specific GAD-cytotoxic T-cell clones and β-cell damage (2,6,7,10,45,46).

The above data could also be consistent with toxicity rendered directly upon the β-cell (7,17,18,31,34), as well as with the view that initial antigen-target tissue interactions may require both sustained supply and high doses of AGE to the β-cell in order to cause diabetes (2,6,7,10,12), as immune infiltration and insulitis alone are not necessarily associated with diabetes.

In summary, low-AGE diets may be effective for maintaining the necessary balance against autoreactive T-cell responses, as well as for preventing direct β-cell injury. The mechanistic hypothesis proposed herein is consistent with the recent significant increase in type 1 diabetes (2,6,7,50), in view of the worldwide assimilation of Western-type AGE-enriched dietary patterns (2,6), including those involved in the fetal stages and in early life. If confirmed clinically, dietary AGE restriction may prove an effective, low-cost, noninvasive strategy for diabetes prevention.

ACKNOWLEDGMENTS

We thank Ina Katz for invaluable editorial assistance. We also thank Dr. Radu Dorel for his helpful advise and Min Lu and Chang Yong Lu for technical assistance.

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