Branched-Chain Amino Acid–Enriched Diet: Effects on Insulin Secretion and Cellular Immune Aggression (44529)

L. M. KARABATAS,* L. FABIANO DE BRUNO,* C. PASTORALE,* Y. B. LOMBARDO,† AND J. C. BASABE*,[1]

*Centro de Investigaciones Endocrinológicas, Hospital de Niños Ricardo Gutierrez, Buenos Aires, Argentina; and †Departamento de Ciencias Biológicas, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, Ciudad Universitaria Paraje El Pozo, Santa Fe, Argentina

Abstract. **Several reports have demonstrated that high-protein diets may have beneficial effects on experimental models of diabetes and have raised the possibility that branched-chain amino acids could play a role in these protective effects. We investigated the effect of a normoproteic, branched-chain amino acid–enriched diet (experimental diet) on insulin secretion from C57BL/6N mice transferred with splenocytes from diabetic syngeneic donors. Mice previously fed with the experimental or control diet received three intraperitoneal injections, every other day, of 5×10^7 viable mononuclear splenocytes obtained from control or diabetic donors. Results showed that mice fed with the experimental diet and transferred with "diabetic" splenocytes presented: i) normoglycemia, and (ii) significantly higher levels in both phases of glucose-induced insulin secretion and normal values of arginine-glucose–induced insulin secretion. To evaluate the *in vitro* cellular immune aggression, dispersed mouse islet cells were co-cultured with splenocytes from syngeneic diabetic mice. First, dispersed islet cells from mice on the experimental or control diet were co-cultured with splenocytes from control or diabetic mice on a commercial diet. In the presence of "diabetic" splenocytes, dispersed islet cells from mice on the experimental diet presented a significantly lower *in vitro* cellular immune aggression. On the other hand, "diabetic" splenocytes from mice fed with the experimental diet produced a significantly reduced cellular immune aggression on dispersed islet cells. Our results showed that feeding branched-chain amino acids increased the capacity of β cells to withstand a functional assault and diminished the extent of *in vitro* cellular immune aggression.** [P.S.E.B.M. 2000, Vol 224:159–165]

Insulin-dependent diabetes is a disease that reflects a variety of genetic, environmental, and immunologic factors (1). Among environmental factors, the diet plays a critical role (2–5). Previous studies have demonstrated that high-protein diets may have beneficial effects in reducing hyperglycemia, polyuria, and glycosuria associated with the diabetic state induced by a single diabetogenic dose of streptozotocin (6–8) and increasing insulin secretion in mice injected with multiple low doses of this drug (9).

In a previous study we reported that mice fed a high-protein diet and injected with mononuclear splenocytes from streptozotocin-diabetic syngeneic donors presented higher levels of stimulated insulin secretion (10). However, long-term administration of high-protein diets can promote an impairment in renal function and, in diabetic rats, is associated with increased glomerular damage (11). Thus, additional information is needed concerning cellular and molecular mechanisms by which high-protein diets exert their protective effects on experimental diabetic syndromes.

Plasma concentrations of most amino acids have been reported to show relatively small changes in rats fed a high-protein diet, except for valine, isoleucine, and leucine,

This work was supported by grants from: Fundación A. J. Roemmers, Fondo Cruz del Sur, and BID 802/OC-AR, FONCyT, PMT-PICT0391 from CONICET.

[1] To whom requests for reprints should be addressed at CEDIE, Hospital de Niños R. Gutierrez, Gallo 1330, (1425) Buenos Aires, Argentina. E-mail: cedie@pccp.com.ar

Received September 15, 1999. [P.S.E.B.M. 2000, Vol 224]
Accepted February 22, 2000.

0037-9727/00/2243-0159$15.00/0

whose concentrations markedly increased (12, 13). This last observation raises the possibility that branched-chain amino acids may play a role in the beneficial effects of protein-rich diets on the evolution of diabetes in animals.

The aim of the present experiments was to investigate to what extent the administration of a normoproteic, branched-chain amino acid–enriched diet (BCD) could protect β cells against the functional impairment of insulin secretion in mice transferred with splenocytes from syngeneic diabetic donors. Perifused pancreatic slices stimulated with glucose or arginine (as a nonglucose secretagog) were used to evaluate insulin secretion in splenocyte-recipient mice.

To study the effect of BCD feeding on: i) the ability of dispersed mouse islet cells (IC) to withstand an *in vitro* cellular immune aggression; and ii) the capacity of these splenocytes to generate *in vitro* functional aggression on insulin secretion from IC, these cells were co-cultured with mononuclear splenocytes from diabetic mice (14, 15).

Materials and Methods

Animals. Male C57BL/6N inbred mice weighing 19–22 g were obtained from the Comisión Nacional de Energía Atómica (CNEA) (Buenos Aires, Argentina). Mice were housed initially in a room with controlled temperature (23°C) and a fixed 12:12-hr artificial light:dark cycle. Animals had free access to water and standard laboratory chow (Cargill, Buenos Aires, Argentina).

The protocols of animal use comply with the *Principles of Laboratory Animal Care* (NIH Publication No. 85–23, revised 1985). Studies were conducted in a jurisdiction where review of the Institution's Animal Care Commitee was not required.

Diets. Two different diets were used: i) a home-made normoproteic, branched-chain amino acid–enriched diet (BCD) (16) consisting of (w/w): 18.00% casein, 52.42% corn starch, 8.00% corn oil, 7.00% cellulose, 3.01% valine, 1.97% isoleucine, 4.10% leucine, 0.20% choline chloride, 0.30% DL-methionine (Sigma, St. Louis, MO), 3.50% salt mixture, and 1.00% vitamin mixture; and ii) a home-made balanced control diet (CD) composed of (w/w): 20.00% casein, 59.50% corn starch, 8.00% corn oil, 7.00% cellulose, 0.20% choline chloride, 0.30% DL-methionine, 3.50% salt mixture, and 1.00% vitamin mixture. Both diets were isocaloric (390 kcal/100 g diet) and available *ad libitum*.

Their vitamin content and salt mixture followed the recommendations of the American Institute of Nutrition *ad hoc* Committee on Standards for Nutritional Studies (17). The salt mixture was based on the AIN-76 salt mix (in g/kg of diet: calcium phosphate dibasic, 17.5; magnesium oxide, 0.84; potassium citrate monohydrate, 7.7; potassium sulfate, 1.82; sodium chloride, 25.9; chromium potassium sulfate, 0.019; cupric carbonate, 0.05; potassium iodate, 0.0003; ferric citrate, 2.1; manganese carbonate, 0.1225; sodium selenite, 0.0003; and zinc carbonate, 0.056). The vitamin mixture was based on the AIN-76[a] vitamin mix (in mg/kg of diet: vitamin A palmitate (500,000 UI/g), 8.8; vitamin D_3 (400,000 UI/g), 2.75; vitamin E acetate (500 UI/g), 11.0; menadione sodium bisulfate, 0.8; biotin (1%), 22.0; cyanocobalamin (0.1%), 11.0; folic acid, 22.0; niacin, 33.0; calcium pantothenate, 17.6; pyridoxine HCl, 7.7; riboflavin, 6.6; and thiamin HCl, 6.6).

Experimental Designs. *Effect of BCD on insulin secretion from mice injected with mononuclear splenocytes (MS) from control or diabetic syngeneic donor mice.* Recipient syngeneic mice received BCD or CD during 15 days before being transferred with mononuclear splenocytes (MS). Mice fed with commercial chow were used as MS donors. The animals were injected intraperitoneally (ip) with either multiple low doses of streptozotocin (SZ) (40 mg/kg) (Sigma Chemical Co., St. Louis, MO) dissolved in citrate buffer (0.1 *M* trisodium citrate, 0.1 *M* citric acid, pH 4.5) or citrate buffer during 5 consecutive days. The mice were sacrificed by cervical dislocation 15 days after the last injection, and MS were aseptically isolated by shredding the spleens with a steel wire mesh followed by two washes in sterile 154 m*M* NaCl solution (18, 19). The viability of MS was 95–98%, assayed by the Trypan blue exclusion test (20).

Recipient syngeneic mice previously adapted for 15 days to BCD or CD received three injections, every other day, of 5×10^7 viable MS (ip in 0.2 ml of sterile 154 m*M* NaCl solution) obtained from SZ-injected donors (diab.MS) or from citrate buffer–injected donors (ctr.MS). Recipient mice were maintained on their diets for another 15 days and sacrificed by cervical dislocation. The pancreas was quickly removed from each and processed for perifusion as described below. Blood samples from nonfasted mice were obtained by retroorbital sinus puncture. All experiments were carried out between 10 AM and 12 noon.

Perifusion of pancreatic slices. The technique described by Burr *et al*. (21) was used with slight modifications (22). Krebs-Ringer-bicarbonate buffer was used as the perifusion buffer and was supplemented with 1% (w/v) bovine albumin fraction V (Sigma, St. Louis, MO) and 3.3 m*M* glucose. The pH of the buffer, kept under constant 95% O_2, 5% CO_2 gassing was 7.38–7.40. The whole pancreas of a single mouse was cut in small slices; half was perifused using 16.5 m*M* glucose as a stimulus, and the other half stimulated with 20 m*M* arginine-5 m*M* glucose. Samples were collected, after an initial 15-min recuperation period, on 0.25 *M* EDTA in tubes kept at 4°C, and immediately frozen at –20°C. Samples from min 1 and 2 were used for baseline determinations. Stimulus was added to the perifusion buffer between min 3–40. Perifusion flux was 1.8–2.2 ml/min.

Effect of BCD on in vitro *cellular immune aggression.* The cellular immune aggression was evaluated studying insulin secretion by dispersed mouse islet cells (IC)

co-cultured with MS from control or diabetic syngeneic mice.

Effect of BCD feeding on the ability of IC to withstand an* in vitro *cellular immune aggression. Mice were fed for 15 days with BCD or CD, sacrificed by cervical dislocation, and their IC isolated and co-cultured with MS from control or diabetic mice as described below. MS were obtained from singeneic mice (fed a commercial laboratory chow) injected either with multiple low doses of SZ (diab.MS) or with citrate buffer alone (ctr.MS). Spleens from diabetic or control mice were pooled to obtain diab.MS or ctr.MS, respectively. IC were isolated from each single pancreas (from BCD- or CD-fed mice) and co-cultured with diab.MS or ctr.MS.

Effect of BCD feeding on the capacity of MS to inhibit insulin secretion from IC. IC were isolated from normal mice fed a commercial mice chow and pooled for co-culture with MS. The MS were isolated from mice previously fed BCD or CD for 15 days, injected with multiple low doses of SZ or buffer, and fed an additional 15-day period with their previous diet. Therefore, mice were sacrificed, and MS were isolated from each single spleen.

Evaluation of cellular immune aggression. ***Isolation of IC.*** Islets of Langerhans were obtained from the pancreas of collagenase-treated (Sigma, St. Louis, MO) mice by the method of Lacy and Kostianovsky (23). To obtain islet-cell suspensions, freshly isolated islets were subjected to EDTA and trypsin (Sigma) as described by Ono *et al*. (24). IC were suspended in basal Minimal Essential Medium (MEM) (Gibco, Paisley, UK) with Eagle salts supplemented with 10% (v/v) fetal calf serum, 2 m*M* glutamine, 1 m*M* sodium piruvate, 0.814 mg/l nonessential amino acids (Gibco, Paisley, UK), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cell viability was estimated by the Trypan blue exclusion test (20), and only cell suspensions having at least 90% viable cells were employed.

IC stimulation. The IC suspension was placed in 96-well Falcon microtest plates (Becton Dickinson, Oxnard, CA) at 5×10^3 cells/100 μl per well, with the addition of 4×10^5 MS from either control or diabetic mice (in 100 μl), or basal medium (100 μl), and incubated for 18 hr (14, 15). Ten wells were used for each determination. At the end of this period, wells were carefully washed, and the supernatant was collected to assess prestimulatory insulin release. The medium was replaced with 200 μl of basal medium (glucose 5.5 m*M*) or 200 μl stimulatory medium (glucose 16.5 m*M* plus theophylline 5.5 m*M*). Five wells were used for each basal or stimulatory medium. MS remained with cells during both basal and stimulatory periods. Supernatants were withdrawn after 5 min and rapidly frozen for insulin determinations. Islet cell function was expressed as the net insulin secretion (μU/5000 cells) during a 5-min incubation in the presence of basal or stimulatory medium (15). Net basal secretion was given for the 5-min period in basal medium minus prestimulatory secretion, whereas net stimulated secretion was also given for the same 5-min period in stimulatory medium minus prestimulatory secretion.

Results of the cellular immune aggression test (net stimulated secretion) were reproducible with a 3.89% interassay and 4.20% intra-assay coefficient of variation (CV), for both ctr.MS or diab.MS.

Analytical Methods. Serum glucose levels from nonfasted mice were determined using Glicemia Enzimatica Kit (Wiener Lab, Buenos Aires, Argentina). Insulin was determined in perifusion samples and in supernatants from IC-MS cultures by the method of Herbert *et al*. (25). Pork monoiodine ^{125}I-insulin was obtained from CENEXA (Facultad de Medicina, Universidad Nacional de La Plata, Argentina). Rat standard insulin was obtained from Novo Research Laboratories (Bagsvaerd, Denmark). Guinea pig anti-porcine insulin antiserum was sufficiently nonspecific as to allow pork-labeled insulin to be displaced by mouse insulin.

Insulin assay sensitivity was 0.5 μU/ml; intra-assay CV was 8.7%, 6.2%, and 5.1% for 1–5, 5–10, and 10–50 μU insulin/ml determination ranges, respectively; interassay CV were 6.6%, 5.0%, and 5.2% for the given ranges.

Statistical Analysis. To evaluate insulin secretion from perifused pancreatic slices, we integrated the areas under the stimulated-insulin secretion curves: the first secretory peak was integrated between min 3–7 and the second peak between min 9–40 of perifusion.

Results were expressed as mean ± SEM. Statistical significance was determined by ANOVA.

When significance was found, Scheffe's *post hoc* comparisons were made; $P < 0.05$ was accepted as statistically significant.

Results

BCD and Insulin Secretion in Mice Transferred with MS from Diabetic or Control Donor Mice. Before the transfer procedure, mice fed during 15 days with the BCD or the CD presented similar weight gains (mean ± SEM) (BCD: 0.27 ± 0.03 g/day, $n = 15$; CD: 0.28 ± 0.04 g/day, $n = 12$). Pretransfer serum glucose levels were also similar for both groups (Table I).

Transfer of diab.MS caused a significant increase in serum glucose levels in the CD-fed recipient mice, but not in BCD-fed mice (Table I).

Basal insulin secretion from perifused pancreatic slices was similar in mice fed either the BCD or the CD and transferred with ctr.MS (BCD: 48.17 ± 3.43, CD: 48.17 ± 4.72 μU/min/100 mg wet tissue, $n = 6$ in both cases) or diab.MS (BCD: 50.44 ± 3.67, CD: 50.67 ± 2.27 μU/min/100 mg wt, $n = 9$ and 6, respectively, *P*:NS).

Transfer of diab.MS caused significant diminutions in the first and second phases of glucose-stimulated insulin secretion from recipient mice fed the BCD or the CD when compared with ctr.MS-recipient mice (Fig. 1). However, the insulin secretory levels reached in BCD-fed mice transferred with diab.MS were significantly higher compared with those observed in mice fed CD.

Table I. Serum Glucose Levels in Mice Transferred with ctr.MS or diab.MS

	Serum glucose (mg %)	
	CD-fed mice	BCD-fed mice
Pretransfer	177.9 ± 4.1 *n* = 12	175.5 ± 4.6 *n* = 15
Transferred with ctr.MS	181.8 ± 4.7 *n* = 6	185.5 ± 6.2 *n* = 6
Transferred with diab.MS	274.0 ± 12.9[a,b] *n* = 6	175.3 ± 7.8 *n* = 9

Note. Nonfasted serum glucose levels (in mg %) from mice fed control diet (CD) or normoproteic, branched-chain amino acids–enriched diet (BCD) and transferred with "diabetic" or control mononuclear splenocytes (diab.MS or ctr.MS). Pretransfer values correspond to animals fed during 15 days with one of the two diets and before being injected with mononuclear splenocytes from syngeneic donors.
[a] $P < 0.01$ versus mice transferred with ctr.MS and fed the CD.
[b] $P < 0.01$ versus mice transferred with diab.MS and fed the BCD. Results are expressed as mean ± SEM.

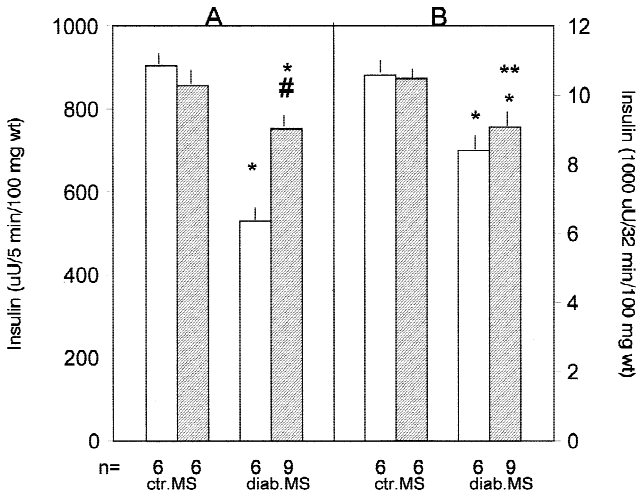

Figure 1. (A) First and (B) second phases of glucose-stimulated insulin secretion (expressed as areas under insulin secretion peaks) in perifused pancreatic slices from recipient mice fed CD □, or BCD ■, transferred with "diabetic" (diab.MS) or control MS (ctr.MS). Results are expressed as mean ± SEM. *$P < 0.01$ vs mice transferred with ctr.MS and fed the same type of diet. #$P < 0.01$ vs mice fed CD and transferred with diab.MS. **$P < 0.05$ vs mice fed CD and transferred with diab.MS.

When perifused pancreatic slices were stimulated with arginine-glucose, the transfer of diab.MS caused an impairment in the first insulin secretory peak only in the CD-fed recipient mice whereas the second secretory peak was not affected (Fig. 2).

Effect of BCD on *In Vitro* Cellular Immune Aggression. *Effect of BCD feeding on the ability of IC to withstand an* in vitro *cellular immune aggression.* IC (without MS) from CD- or BCD-fed mice present similar values of insulin secretion when preincubated 18 hr (IC from CD-fed mice: 2.12 ± 0.01, and IC from BCD-fed mice: 2.12 ± 0.01 μU/18 hr/5000 cells, $n = 10$ and $n = 12$; *P*:NS), or incubated 5 min either in basal conditions (IC from CD-fed mice: 2.21 ± 0.13, and IC from BCD-fed mice:

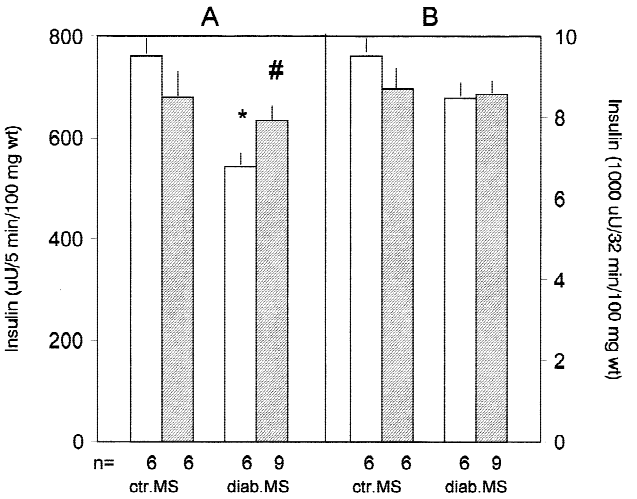

Figure 2. (A) First and (B) second phases of arginine-glucose-stimulated insulin secretion (expressed as areas under insulin secretion peaks) in perifused pancreatic slices from recipient mice fed CD □, or BCD ■, and injected with "diabetic" MS (diab.MS) or control MS (ctr.MS). Results are expressed as mean ± SEM. *$P < 0.01$ vs mice fed CD and injected with ctr.MS. #$P < 0.05$ vs mice fed CD and injected with diab.MS.

2.13 ± 0.11 μU/5 min/5000 cells, $n = 10$ and 12) or in stimulatory conditions (IC from CD-fed mice: 24.59 ± 0.33, IC from BCD-fed mice: 24.54 ± 0.31 μU/5 min/5000 cells, $n = 10$ and 12, respectively).

Insulin secretion shows similar values when IC from CD- or BCD-fed mice were co-cultured with ctr.MS or diab.MS both in the prestimulatory period of 18 hr (IC from CD-fed mice: with ctr.MS: 2.15 ± 0.01, with diab.MS: 2.13 ± 0.01; IC from BCD-fed mice: with ctr.MS: 2.14 ± 0.01, with diab.MS: 2.12 ± 0.01 μU/18 hr/5000 cells, $n = 5$, 5, 6, and 6) or in basal conditions (IC from CD-fed mice: with ctr.MS: 2.26 ± 0.07, with diab.MS: 2.23 ± 0.07; IC from BCD-fed mice: with ctr.MS: 2.15 ± 0.08, with diab.MS: 2.28 ± 0.24 μU/5 min/5000 cells, $n = 5$, 5, 6, and 6, respectively).

When IC from CD- or BCD-fed mice were incubated with diab.MS under stimulatory conditions, a diminished insulin secretion was observed, compared with IC incubated with ctr.MS. However, this diminution was significantly less severe for IC from BCD-fed mice (Fig. 3).

Effect of BCD feeding on the capacity of MS to inhibit insulin secretion from IC. Insulin secretion presented similar values when IC from mice fed a commercial chow were preincubated 18 hrs, or incubated 5 min in basal conditions with ctr.MS or diab.MS (data not shown).

When IC were incubated under stimulatory conditions with diab.MS from CD- or BCD-fed mice, there was a decrease in insulin secretion. However, this decrement was significantly less severe when MS came from BCD-fed mice (Fig. 4).

Discussion

A growing body of recent evidence indicates that high-protein diets may have beneficial effects on streptozotocin-

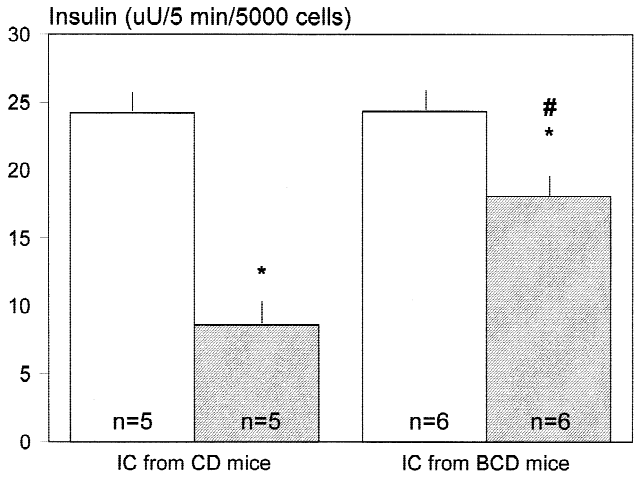

Figure 3. Net stimulated insulin secretion of IC from CD- or BCD-fed mice, cultured with ctr.MS or diab.MS. *P < 0.01 vs IC cultured with ctr.MS. #P < 0.01 vs IC from CD-fed mice cultured with diab.MS.

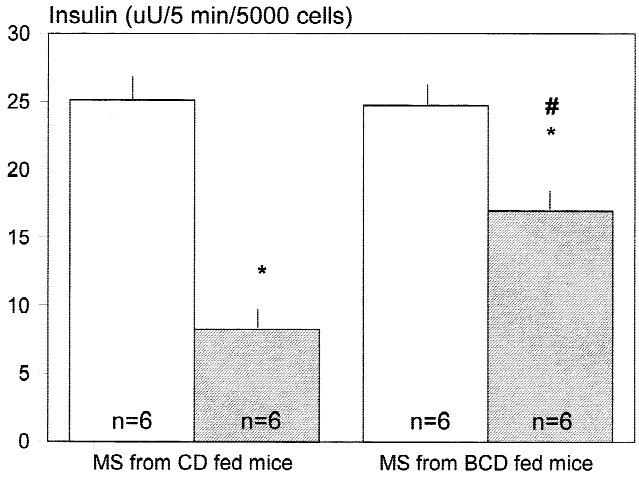

Figure 4. Net stimulated insulin secretion from IC cultured with ctr.MS or diab.MS obtained from mice fed CD or BCD. *P < 0.01 vs IC cultured with ctr.MS from CD- or BCD-fed mice, respectively. #P < 0.01 vs IC cultured with diab.MS from CD-fed mice.

induced diabetes in rats and mice (6, 7, 9). Yet, long-term administration of high-protein diets have been associated with increased renal injury in diabetic rats (11). Previous studies with animals on protein-rich regimens have shown that plasma levels of branched-chain amino acids were increased (12, 13). Eizirik *et al*. (16) reported that the amount of branched-chain amino acids supplied by BCD was sufficient to produce plasma branched-chain amino acid levels similar to those observed in animals on a high-protein diet.

It has been reported that rats adapted 15 days to BCD, and injected with a single diabetogenic dose of streptozotocin, showed a significant reduction in the severity of diabetes, suggesting that the increased supply of branched-chain amino acids is responsible, at least partly, for the previously reported beneficial effects of high-protein diets in streptozotocin-induced diabetes (16).

Eizirik *et al*. have suggested that high-protein diets and BCD can exert these beneficial effects by an initial protection against the diabetogenic action of streptozotocin at the time of exposure (7, 13, 16). Isolated mouse pancreatic islets (pre-incubated with a mixture of branched-chain amino acids) exposed *in vitro* to streptozotocin showed a smaller decrease in islet number, an increased total islet DNA content, and a higher insulin release (26).

In the present work we investigated the effect of a normoproteic, branched-chain amino acid–enriched diet (BCD) administration on the MS transfer from diabetic mice to syngeneic recipients (18), an experimental model of diabetes without the toxic action of streptozotocin. Arata *et al*. (19) showed that when 5×10^7 MS from diabetic donors were injected into syngeneic healthy mice, the recipient animals did not present basal hyperglycemia but had abnormal intraperitoneal glucose tolerance and a diminished glucose-induced insulin secretion. Also, "diabetic" MS were preferentially trapped by the recipient's pancreas, and both donor and recipient T lymphocytes played an important part in the transfer-related alterations (19).

In a previous study, we showed that a high-protein diet has protective and restorative effects on the insulin secretion patterns from this experimental model where immune aggression (and not a direct toxic effect of streptozotocin) was present, since recipient mice fed this diet and injected with MS from syngeneic diabetic donors showed partial restoration of stimulated insulin secretion (10).

In this study we used three injections of "diabetic" MS to enhance the immune aggression in recipient mice. Our results indicated that mice fed CD and injected three times with 5×10^7 diab.MS responded with hyperglycemia and significant diminutions in glucose-induced and arginine-induced insulin secretion. However, when recipient mice were fed the BCD, they responded with normoglycemia, higher levels in glucose-stimulated insulin secretion, and a normal response to arginine.

The BCD has a somewhat higher total nitrogen content compared with the CD. Because branched-chain amino acids are metabolized and the resulting nitrogen enters the nonspecific nitrogen pool, the possibility cannot be ruled out that in addition to the specific effects, these amino acids could also have nonspecific effects *via* contributing nitrogen.

Both BCD and CD were casein-based diets since the source of dietary protein can be a major component in the modulating effect of diet upon autoimmune diabetes as described in the BioBreeding (BB) rat and non-obese diabetic (NOD) mouse (2, 4, 5, 27).

In our experimental conditions, our results showed, in agreement with Harper *et al*. (28), that the supplementation of a normoproteic diet with the three branched-chain amino acids did not induce any demonstrable deleterious effects, and that quantities of many amino acids that cause growth depressions in animals fed a low-protein diet may be quite well tolerated by animals fed a diet adequate in protein. The National Academy of Science's report on maximum possible weight gain in 26 inbred male mouse strains indicated

that, at a starting weight of 21 g, the average weight gain from age 6–8 weeks was 0.22 g/day (29). This value is comparable to our weight gains from CD- and BCD-fed mice.

Since the effects of the BCD on the diabetic state induced by the transfer of diab.MS can be exerted on the immune system or on the β cells, we tested the effect of the BCD on i) the capacity of MS to inhibit insulin secretion from IC and ii) the capacity of IC to withstand a functional aggression.

We used the term immune aggression to indicate that splenocytes from diabetic donors are able to diminish insulin secretion from dispersed islet cells.

Data from previous studies showed that peripheral blood mononuclear cells from newly diagnosed insulin-dependent diabetic patients developed *in vitro* cellular immune aggression (CIA) toward pancreatic β cells (30). Furthermore we used CIA together with insulin autoantibodies as well as HLA antigens and residual C-peptide to determine whether an immune mechanism could be operating in type 2 diabetic patients with secondary failure to oral hypoglycemic agents (15). Also, splenocytes from mice rendered diabetic by multiple low-dose streptozotocin treatment were found to inhibit insulin secretion from dispersed rat islet cells (31).

The inhibitory effects of mononuclear cells appear to be specific for pancreatic β cells since no cytotoxicity is observed against fibroblasts; furthermore, secretion of glucagon and somatostatin remain unaffected (32). Such inhibition of insulin secretion is not altered by insulin treatment or the presence of antibody, complement, or aggregated IgG (33). Cytoadherence of diabetic lymphocytes is increased in two xenogeneic species (rat and hamster), but not in seven noninsulin-secreting cell lines (33, 34).

Our data showed that IC co-cultured with diab.MS from CD-fed mice produced a significant diminution in stimulated insulin secretion. However, when diab.MS were obtained from BCD-fed mice, the cellular immune aggression was significantly lower (IC released higher levels of insulin). These results indicated that, under our experimental conditions, immune cells from BCD-fed animals had a reduced aggressive capacity.

It has been reported that an excess of dietary leucine in rats diminished the production of rosette-forming cells in lymph nodes and spleen (35) and that the addition of leucine in the diet of growing lambs reduced antibody production and lymphocyte blastogenesis (36). Furthermore, administration of branched-chain amino acids decreased the severity of the diabetic state induced in mice by the encephalomyocarditis (EMC) virus, producing lower glucose levels and islet lymphocytic infiltration (37).

Dietary constituents can influence immune reactivity both in the BB rat and the NOD mouse (2, 4, 5, 27, 38). In the BB rat, the administration of a hydrolyzed casein-based diet prevented the hyperexpression of MHC class I molecules on β cells (39), and the islets of Langerhans showed fewer infiltrating cells and a Th2 cytokine pattern (5).

Our results also showed that when IC from BCD-fed mice were co-cultured with diab.MS, the reduction in stimulated insulin secretion was attenuated. These *in vitro* results correlate to our *in vivo* situation, since mice fed BCD and transferred with diabetic splenocytes presented normoglycemia, higher levels in both phases of glucose induced-insulin secretion and normal response to arginine-glucose. These results suggest an increased capacity of β cells to withstand a functional assault both *in vitro* and *in vivo*.

The addition of leucine in long-term isolated islet cultures was reported to exert a beneficial effect on cell function, especially insulin biosynthesis (40) and to increase NADPH:NADP and NADH:NAD ratios in islets, augmenting the defense mechanism against oxidative damage (41).

The possible extrapancreatic effects of branched-chain amino acids must also be considered. In skeletal muscle, these amino acids serve as energy substrate and possess special anticatabolic effects stimulating protein synthesis and reducing protein breakdown (42).

In conclusion, our results indicated that the effective dietary prevention in the diabetic state induced by three injections of diab.MS may be due to a reduced functional damage of β cells. The diet may also participate as an important factor determining the recovery and regeneration of the β cells.

Immunity or autoimmunity inflammation has been modulated successfully by varying the amount and quality of dietary constituents. Animal studies proved that dietary factors contribute to the prevention of type 1 diabetes (2), and this could provide useful approaches to analogous studies in humans. The possibility that human type 1 diabetes might be prevented by certain dietary constituents early in life is an attractive concept. However, presently, the knowledge is only partially developed and will require more investigation before such recommendations can be made with some hope of success.

We gratefully acknowledge the excellent technical assistance of Mrs. Susana Santillo and Dr. Myriam A. de Rodi.

1. Castaño L, Eisenbarth G. Type I diabetes: A chronic autoimmune disease of human, mouse, and rat. Annu Rev Immunol **8:**647–679, 1990.
2. Scott FW, Elliott RB, Kolb H. Diet and autoimmunity: Prospects of prevention of Type I diabetes. Diabetes Nutr Metab **2:**61–73, 1989.
3. Riccardi G, Rivellese AA. Can diabetes mellitus be prevented by diet? Diabetes Nutr Metab **2:**259–262, 1989.
4. Scott FW. Food-induced type 1 diabetes in the BB rat. Diabetes Metab Rev **12:**341–359, 1996.
5. Scott FW, Cloutier HE, Kleemann R, Woerz-Pagenstert U, Rowsell P, Modler HW, Kolb H. Potential mechanisms by which certain foods promote or inhibit the development of spontaneous diabetes in BB rats: Dose, timing, early effects on islet area, and switch in infiltrate from Th1 to Th2 cells. Diabetes **46:**589–598, 1997.
6. Siegel EG, Trapp VE, Wollheim CB, Renold AE, Schmidt FH. Beneficial effects of low-carbohydrate–high-protein diets in long-term diabetic rats. Metabolism **29:**421–428, 1980.
7. Eizirik DL, Migliorini RH. Reduced diabetogenic effect of streptozotocin in rats previously adapted to a high-protein, carbohydrate-free diet. Diabetes **33:**383–388, 1984.

8. Eizirik DL, Tze WJ, Tai J, Migliorini RH. Effects of a high-protein diet on the evolution of diabetes in streptozotocin-induced and spontaneously diabetic BB Wistar rats. Acta Diabetol Lat **23:**107–116, 1986.
9. Karabatas LM, Lombardo YB, Basabe JC. High-protein diet: Effect on insulin secretion patterns from streptozotocin-diabetic rats and mice. Acta Physiol Pharmacol Ther Latinoam **42:**239–254, 1993.
10. Karabatas LM, Lombardo Y, Basabe JC. Effect of high-protein feeding on insulin secretion in mice injected with splenocytes from diabetic mice. Proc Soc Exp Biol Med **211:**81–86, 1996.
11. Reyes AA, Klahr S. Dietary supplementation of L-arginine ameliorates renal hypertrophy in rats fed a high-protein diet. Proc Soc Exp Biol Med **206:**157–161, 1994.
12. Eisenstein AB, Strack I, Galo-Torres H, Georgiadis A, Miller ON. Increased glucagon secretion in protein-fed rats: Lack of relationship to plasma amino acids. Am J Physiol **236:**E20–E27, 1979.
13. Eizirik DL, Kettelhut IC, Migliorini RH. Administration of branched-chain amino acids reduces the diabetogenic effect of streptozotocin in rats. Braz J Med Biol Res **20:**137–144, 1987.
14. Boitard C, Debray-Sachs M, Plouplard A, Assan R, Hamburger J. Lymphocytes from diabetics suppress insulin release *in vitro*. Diabetologia **21:**41–46, 1981.
15. Zavala AV, Fabiano de Bruno LE, Cardoso AI, Mota AH, Capucchio M, Poskus E, Fainboim L, Basabe JC. Cellular and humoural autoimmunity markers in Type 2 (noninsulin-dependent) diabetic patients with secondary drug failure. Diabetologia **35:**1159–1164, 1992.
16. Eizirik DL, Germano CM, Migliorini RH. Dietetic supplementation with branched-chain amino acids attenuates the severity of streptozotocin-induced diabetes in rats. Acta Diabetol Lat **25:**117–126, 1988.
17. Bieri JG. Second report of the *ad hoc* Committee on Standards for Nutritional Studies. J Nutr **110:**1726, 1980.
18. Buschard K, Rygaard J. Passive transfer of streptozotocin-induced diabetes mellitus with spleen cells. Acta Pathol Microbiol Scand (C) **85:**469–472, 1977.
19. Arata M, Fabiano de Bruno L, Goncalvez Volpini W, Quintans JC, D'Alessandro VG, Braun M, Basabe JC. β-cell function in mice injected with mononuclear splenocytes from multiple-dose streptozotocin diabetic mice. Proc Soc Exp Biol Med **206:**76–82, 1994.
20. Phillips HJ. Dye exclusion test for cell viability. In: Kruse G, Patterson C, Eds. Tissue: Methods and Applications. New York: Academic Press, pp406–408, 1973.
21. Burr IM, Stauffacher W, Balant L, Renold AE, Grodsky G. Dynamic aspects of proinsulin release from perifused rat pancreas. Lancet **2:**882–883, 1969.
22. Basabe JC, Karabatas LM, Arata M, Pivetta OH, Cresto JC. Secretion and effect of somatostatin in early stages of the diabetic syndrome in C57BL/KsJ mdb mice. Diabetologia **29:**485–488, 1986.
23. Lacy PE, Kostianovsky M. Method for the isolation of intact islets of Langerhans from the rat pancreas. Diabetes **16:**35–39, 1967.
24. Ono J, Takaki R, Fukuma M. Preparation of single cells from pancreatic islets, of adult rats by the use of dispase. Endocrinol Jpn **24:**265–270, 1977.
25. Herbert V, Lau K, Gottlieb CW, Bleicher SJ. Coated charcoal immunoassay of insulin. J Clin Endocrinol Metab **25:**1375–1384, 1965.
26. Eizirik DL, Sandler S. The partial protective effect of branched chain amino acids against streptozotocin-induced cytotoxicity to mouse pancreatic islets *in vitro*. Pharmacol Toxicol **61:**320–324, 1987.
27. Scott FW, Mongeau R, Kardish M, Hatina G, Trick KD, Wojcinski Z. Diet can prevent diabetes in the BB rat. Diabetes **34:**1059–1062, 1985.
28. Harper AE, Benevenga NJ, Wohlueter RM. Effects of ingestion of disproportionate amounts of amino acids. Physiol Rev **50:**428–558, 1970.
29. National Research Council. Nutrient requirements of the mouse. In: National Research Council, Ed. Nutrient Requirements of Laboratory Animals (3rd ed). Washington, DC: National Academy of Sciences, pp38–53, 1978.
30. Arata M, Fabiano de Bruno L, Goncalvez Volpini W, Gagliardi G, Quintans CJ, Basabe JC. Insulin secretion by pancreas of athymic mice injected with peripheral mononuclear cells from insulin-dependent diabetic patients. Metabolism **44:**1435–1441, 1995.
31. Karabatas LM, Fabiano de Bruno L, Pastorale CF, Cullen C, Basabe JC. Inhibition of nitric oxide generation: Normalization of *in vitro* insulin secretion in mice with multiple low-dose streptozotocin and in mice injected with mononuclear splenocytes from diabetic syngeneic donors. Metabolism **45:**940–946, 1996.
32. Boitard C, Chatenoud L, Debray-Sach N. *In vitro* inhibition of pancreatic β cell function by lymphocytes from diabetics with associated autoimmune diseases: A T-cell phenomenon. J Immunol **129:**2529–2531, 1982.
33. Lang F, Maugendre D, Houssaint E, Charbonnel B, Sai P. Cytoadherence of lymphocytes from type I diabetic subjects to insulin-secreting cells: Marker of anti-β-cell cellular immunity. Diabetes **36:**1356–1364, 1987.
34. Segain JP, Valentin A, Bardet S, Feve B, Sevestre H, Houssaint E, Charbonnel B, Sai P. *In vitro* relationship of CD4 cells from type I diabetic patients and xenogeneic β-cell membranes. Diabetes **38:**634–640, 1989.
35. Aschkenasy A. Differing effects of dietary protein deprivation on the production of rosette-forming cells in the lymph nodes and the spleen and on the level of serum hemagglutinin in rats immunized to sheep red blood cells. Immunology **24:**617–633, 1973.
36. Kuhlman G, Roth JA, Flakoll PJ, Vandehaar MJ, Nissen S. Effects of dietary leucine, α-ketoisocaproate and isovalerate on antibody production and lymphocyte blastogenesis in growing lambs. J Nutr **118:**1564–1569, 1988.
37. Utsugi T, Kanda T, Tagima Y, Kato N, Tomondo S, Suzuki T, Kawazu S, Murata K. Branched chain amino acids suppress the diabetogenicity of a new variant of EMC virus. Diabetes **40**(Suppl 1)**:**216A, 1991.
38. Elliott RB, Reddy SN, Bibby NJ, Kida K. Dietary prevention of diabetes in the non-obese diabetic mouse. Diabetologia **31:**992–998, 1994.
39. Li XB, Scott FW, Park YH, Yoon JW. Low incidence of autoimmune type I diabetes in BB rats fed a hydrolised casein-based diet associated with early inhibition of non-macrophage-dependent hyperexpression of MHC class I molecules on β cells. Diabetologia **38:**1138–1147, 1995.
40. Schafer G, Schatz H. Long-term effects of leucine and arginine on B-cell function of cultivated pancreatic islets. J Endocrinol **91:**255–262, 1981.
41. Ammon HPT, Hoppe E, Akhtar MS, Niklas H. Effect of leucine on the pyridine nucleotide contents of islets and on the insulin release: Interactions *in vitro* with methylene blue, thiol oxidants, and p-chloromercuribenzoate. Diabetes **28:**593–599, 1979.
42. Madsen DC. Branched-chain amino acids: Metabolic roles and clinical application. In: Johnston JDA, Ed. Advances in Clinical Nutrition: Proceedings of the 2nd International Symposium. Boston: MTP Press Limited, pp3–23, 1982.