Protein-Free Diets Do Not Protect High-Incidence Diabetes-Prone BioBreeding Rats From Diabetes

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Dietary factors have been reported to affect the development of spontaneous diabetes in various colonies of inbred and outbred diabetes-prone (DP) BioBreeding (BB) rats. Several studies have attributed a protective effect to a diet omitting crude protein mixtures in favor of purified casein, hydrolyzed casein, or free amino acids. We have used inbred BB rats, all of which become diabetic in specific pathogen-free (SPF) conditions when fed ordinary rat chow, to test the capacity of 2 different protein-free diets to modulate BB rat diabetes in 2 distinct pathogen-free environments. BB rats known to all develop diabetes by 100 days of age were fed from birth with 1 of 3 diets. By 120 days of age, 100% of the animals on a standard diabetogenic chow diet, 83% of animals on an amino acid-based protein-free diet, and 100% of animals on a hydrolyzed casein-based diet had developed diabetes ($P > .05$). A slight delay in the age of onset was observed among rats fed the amino acid-based diet, but this delay coincided with a reduction in weight gain among these animals compared with the rats on a standard diet. Histology showed insulitis in all rats at either diabetes onset or 120 days of age. We conclude that our unique strain of specific pathogen-free BB rats are not protected from diabetes when fed an amino acid-based diet and suggest that their insensitivity to dietary manipulation may be due to an as yet unknown factor present in the diabetes-resistant (DR), but not the DP BB rat genetic background.

INSULIN-DEPENDENT DIABETES mellitus (IDDM) is an autoimmune disease directed against the β cells of the pancreatic islets. The consequent reduced production of insulin leads to hyperglycemia, ketosis, weight reduction, and insulin dependence. Spontaneous diabetes in the nonobese diabetic (NOD) mouse and diabetes-prone (DP) BioBreeding (BB) rat closely resembles human IDDM[1] and allows these animals to be useful for investigating IDDM etiology and pathogenesis. The spontaneously diabetic BB rat is a particularly robust model, because diabetes is controlled by only 3 genes,[2,3] and 100% of the animals develop diabetes, regardless of gender, when maintained in specific pathogen-free (SPF) conditions.[4] Insulitis develops spontaneously at about 50 days,[5] and following the failure to gain weight, clinical diabetes ensues in 1 to 3 days.[6] The event is characterized by the rapid infiltration of the islets of Langerhans by macrophages, T cells, and B cells.[7]

Several lines of evidence have demonstrated that ennvironmental factors can regulate the expression of diabetogenic genes in the BB rat. In particular, diet modifications have been shown to influence diabetes incidence (Table 1). While a high proportion of BB rats fed a standard diet containing a complex crude protein mixture become diabetic, the removal of dietary proteins derived from wheat gluten,[8] soybeans,[9] or cow's milk[10] has been variably linked to a reduced occurrence of IDDM. The substitution of all dietary proteins with hydrolyzed casein or free L-amino acids has achieved the largest and most consistent protective effect, with a reduction in disease frequency ranging between 48%[11] and 100%.[12] Importantly, the protective effect of protein-free diets was observed in colonies of both inbred and outbred BB rats. However, with the exception of one study,[13] the most consistent and spectacular effects of diet on IDDM incidence was observed in outbred animals maintained either in SPF or in open environments.

The mechanisms through which diet can protect from IDDM remain unknown, but there is indirect evidence that diet may act at the level of β-cell antigenicity. Several immune abnormalities of the BB rat are modified by a protective diet, including major histocompatibility complex (MHC) class I antigen hyperexpression on β cells[14] and a Th1 pancreatic cytokine profile,[15] both characteristic of an anti–β-cell inflammatory response. Based on available data, investigators have proposed that exposure of DP rats to one or more specific dietary proteins predisposes to inflammation, in general, and to islet-specific autoimmune response, in particular, possibly via antigen mimicry. A similar sequence of events has also been suggested to occur in both celiac and Crohn's diseases.[16,17] The DP BB rat exhibits an increased gastrointestinal permeability similar to that seen in celiac and Crohn's disease patients.[18] This increased permeability, which develops well before the onset of diabetes, could allow luminal protein antigens to penetrate the epithelial barrier of the intestinal tract and interact with the underlying mucosal immune tissues.

Our laboratory developed the DR.lyp rat by introgression of the *lyp* region onto the diabetes-resistant (DR) BB background through marker-assisted interbreeding, producing a DP strain that differs from the DR BB rat only at the *lyp* locus.[5] These animals develop diabetes in a highly consistent fashion, between 60 and 90 days of age at a frequency exceeding 95%[19] and permit the mapping of *lyp* and other diabetogenic loci.[2,3] As previous studies of diet responses used BB rats with a rather low and highly variable frequency of diabetes incidence under standard conditions, we decided to analyze the protective effect of a protein-free diet in uniformly diabetic BB rats kept in SPF conditions. Our hypothesis was that such an analysis would

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Table 1. Results of Earlier Studies on the Effect of Diet on the Cumulative Incidence of Diabetes in the BB Rat

Year	Diet Protein Source	Age at Diet Exposure	% IDDM Frequency	% Reduction *v* Chow	Weight Gain Difference	Reference
1984	L-amino acids	16 days	15 (3/19)	69	Chow-fed rats grew significantly faster	8
	amino acids + 1% gliadin	16 days	35 (7/20)	29		
	amino acids + 1% milk powder	16 days	52 (11/21)	0		
	Chow	Birth	49 (19/39)			
1984	Casein	21 days	10 (2/20)	75	None	9
	Wheat gluten	21 days	40 (8/20)			
1985	Casein	Unknown	0 (0/40)	100	Casein-fed rats grew significantly faster	10
	Chow	Unknown	28 (11/40)			
1991	Hydrolyzed casein	Birth	12 (3/25)	82	None	11
	Soybean meal	Birth	47 (7/15)	31		
	NIH-07 chow	Birth	68 (17/25)			
1992	Casein	19–33 days	24 (5/21)	64	Chow-fed males grew significantly faster	12
	Altromin chow	Birth	67 (14/21)			
1995	Hydrolyzed casein	Weaning	18 (4/22)	76	None	13
	Purina chow	Birth	76 (16/21)			
1997	Hydrolyzed Casein	Birth	40 (14/35)	54	None	14
	Hydrolyzed casein (NutramIgen)	Birth	45 (14/31)	48		
	Total milk protein	Birth	44 (15/34)	49		
	Purina chow	Birth	87 (54/62)			
1997	Hydrolyzed casein	23 days	11 (22/202)	82	None	15
	Casein	23 days	12 (28/231)	81		
	NIH-07 chow	23 days	62 (120/193)			
1997	Hydrolyzed casein	Weaning	20 (3/14)	60	None	16
	Purina chow	Weaning	50 (7/14)			
Present study	L-amino acids	Birth	87 (10/12)	13	Chow-fed rats grew significantly faster	
	Pregestimil	Birth	100 (12/12)	0		
	Purina chow	Birth	100 (23/23)			

provide a definitive measure of the protective effect of a protein-free diet.

RESEARCH DESIGN AND METHODS

Animals

Viral antibody-free DP BB/Wor rats were obtained from Biomedical Research Models (Worcester, MA). Female animals used for breeding were protected from diabetes by a single transfusion of spleen cells derived from DR BB/Wor donors as previously described,[20] a treatment that increases offspring viability, but has no effect on IDDM frequency in offspring. DP BB/Wor females were mated to diabetic DR.lyp BB males from the University of Washington BB rat colony. The DR.lyp diabetic males, from our congenic line of DR.lyp animals produced by marker-assisted interbreeding, differ from the DR line only at the *lyp* locus.[5] All animals, parents, and offspring are, therefore, homozygous for the *lyp* allele. The frequency of spontaneous IDDM in male and female DR.lyp and DP BB/Wor rats exceeds 95% by 100 days of age when fed a standard chow diet and maintained in SPF conditions. All parents and offspring used in this study were certified to be viral antibody free.

Diets

University of Washington. At birth, 2 litters (n = 12) and their dams were switched from the standard Purina rodent chow (Harlan Sprague Dawley, Madison, WI) to a purified diet in which proteins were replaced by a mixture of free L-amino acids (18% of diet by weight). The diet (Harlan-Teklad, Madison, WI) also contained sucrose (49%), corn starch (15%), corn oil (10%), cellulose (3%), and AIN-76 mineral and vitamin mixes. With the exception of the mode of protein replacement, this diet is identical to the AIN-76A casein-based purified diet used in several previous experiments.[8,9,11-13,15] The amino acid component is essentially similar to that of a diet containing hydrolyzed casein fragments; most of the amino acids in the commercial hydrolyzed casein diets, Nutramigen and Pregestimil, are free, and the remainder exist in small hypoallergenic peptides less than 1,500 dalton in weight.[21] As a control, 2 more F1 DR.lyp × BB DP/Wor litters (n = 12) were kept on the standard Purina rodent chow, in which protein sources included fish meal, wheat gluten, soybean meal, and whey. Animals were weaned from their mothers at 30 days of age and were allowed free access to water and their respective diets at all times.

Diets were checked for protein content by Bradford assay (Sigma, St Louis, MO), using 300 mg of pellet from each diet. Ground pellet was homogenized in 6 mL extraction buffer (150 mmol/L NaCl, 25 mmol/L Tris, 5 mmol/L EDTA, 10% glycerol, 10 μg/mL aprotenin, 1 mmol/L benzamidine), precipitated with 0.07% beta-mercaptoethanol (B-ME) in acetone overnight at 20°C, washed in ice-cold acetone, and redissolved in 3 mL extraction buffer. A total of 10 μL of each sample was then reacted with 200 μL Bradford reagent for 5 minutes, and the 650 nm absorbance of each sample in triplicate was read on a ThermoMax microplate reader (Molecular Devices, Sunnyvale, CA).

University of Toronto. To eliminate the potential effects of maternal transmission of dietary antigens to pups, pregnant females were maintained on Pregestimil (Mead Johnson, Belleville, Ontario, Can-

ada), a hydrolyzed casein-based diet, or Purina rodent chow (Harlan Sprague Dawley) during gestation and nursing. Ingredients of the Pregestimil diet include hydrolyzed casein (14% of diet by weight), medium chain triglycerides (MCT) oil (15.4%), corn oil (5.6%), soy oil (3.5%), sunflower oil (3.5%), corn syrup solids (34%), modified corn starch (9.9%), dextrose (5.6%), citrates (1.13%), and mineral and vitamin mixes. Pups (n = 7 for both diets) were weaned on their dams' diet and allowed free access to water and food. As controls, 2 litters of DP BB/Wor rats were fed Pregestimil (n = 5) or Purina rat chow (n = 4).

Diagnosis of Diabetes

University of Washington. Animals were weighed daily from 50 days of age and checked for high blood glucose levels if they did not gain weight. A rat exhibiting a blood glucose concentration greater than 240 mg/dL on 2 consecutive days was considered diabetic and killed the second day. All rats remaining diabetes-free at 120 days of age were killed by exsanguination through heart puncture under ketamine/xylazine anesthesia.

University of Toronto. Animals were weighed weekly from the age of 30 days and checked for the presence of glycosuria (Tes-Tape; Eli Lilly, Toronto, Ontario, Canada) 3 times a week from 50 days of age. Animals that were glycosuric on 2 consecutive days were tested for hyperglycemia (>240 mg/dL) and killed if diabetic. The pancreas of all animals was removed and processed for histologic examination.

Histology. The pancreas was fixed in 4% paraformaldehyde, paraffinized, and cut into 5-μm sections. Sections were stained with hematoxylin and eosin and scored for insulitis by separate blinded investigators on a scale of 0 (healthy islets) to +4 (end-stage insulitis) as described.[19]

RESULTS

Diet Protein Content

The total protein concentration of the amino acid-based diet homogenate was found to be 3.6 ± 7.0 μg/mL, while that of the Purina chow was 1,660 ± 262 μg/mL.

Diabetes development. All of the F1 DR.lyp × DP BB/Wor rats on the Purina chow (12/12 at the University of Washington and 7/7 at the University of Toronto), 83% (10/12) of those on the amino acid-based chow, and 100% (7/7) of those fed Pregestimil developed diabetes by 120 days of age. All DP BB/Wor rats fed standard diabetogenic chow or Pregestimil (n = 4 and 6, respectively) also developed diabetes. The average age of onset was 73 days among Purina-fed F1 animals at the University of Washington compared with 79 days in the amino acid-based diet group. At the University of Toronto, F1 animals on the Purina diet developed diabetes at an average of 84 days, while F1 animals on the Purina diet developed diabetes at an average of 77 days. Log-rank statistical analysis of the Kaplan-Meier survival curves (Fig 1A and B) showed that there was not a significant difference between the 4 groups. Diabetes onset in BB DP/Wor animals was significantly accelerated by the feeding of a Pregestimil diet; average ages of onset were 91 and 101.5 days for the Pregestimil and Purina diets, respectively. Log-rank analysis of the Kaplan-Meier survival curve (Fig 1C) yielded a *P* value of .0067.

Weight progression. At the University of Washington, animals on the amino acid-based diet initially gained weight at a significantly lower rate than rats on the Purina diet (Fig 2A and B). At 55 days of age, animals on the Purina diet weighed

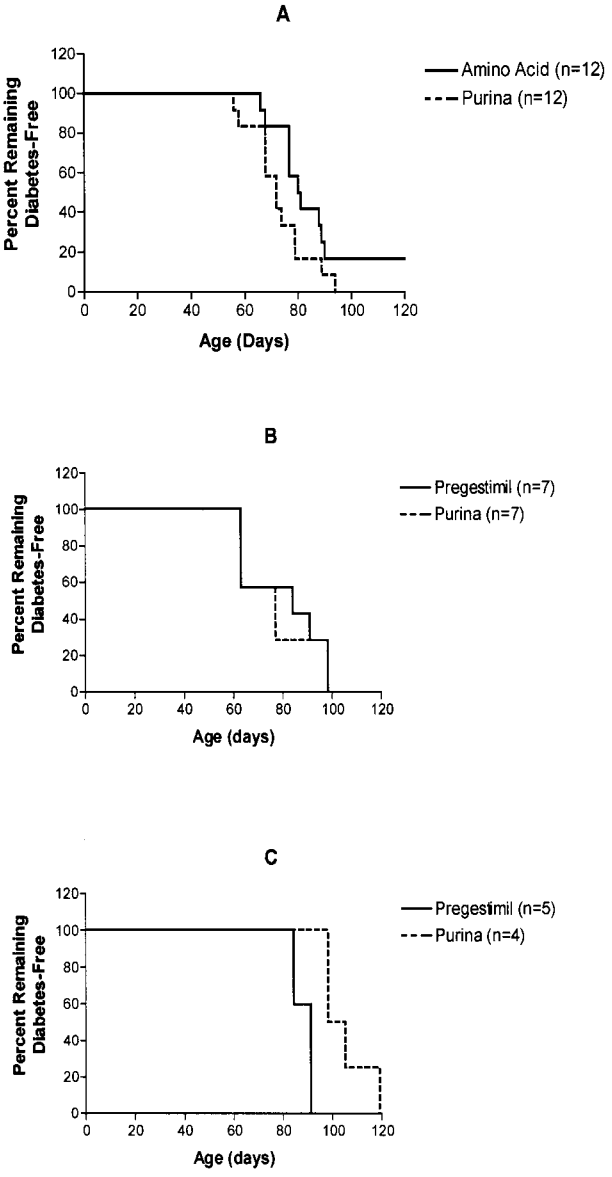

Fig 1. Cumulative survival by age and diet. No reduction in frequency or delay in onset of diabetes was observed among rats fed a protein-free diet. (A) F1 (DR.lyp × BB DP/Wor) rats kept at the University of Washington. (B) F1 (DR.lyp × BB DP/Wor) rats kept at the University of Toronto. (C) BB DP/Wor rats kept at the University of Toronto.

208 ± 18 g (males) and 155 ± 15 g (females) (mean values ± SD). At the same age, rats on the amino acid-based diet averaged 192 ± 9 g (males) and 145 ± 10 g (females). At 70 days of age, the slight weight disparity had disappeared: chow-fed males and females averaged 243 ± 28 g and 172 ± 16 g, respectively, while test diet-fed rats averaged 248 ± 13 g and 170 ± 9 g. At the University of Toronto, the feeding of Pregestimil was also associated with slower initial weight gain (Fig 2C through F). In all 3 groups and among both males and females, analysis of covariance (ANCOVA) analysis showed a significant difference between elevations of the linearized

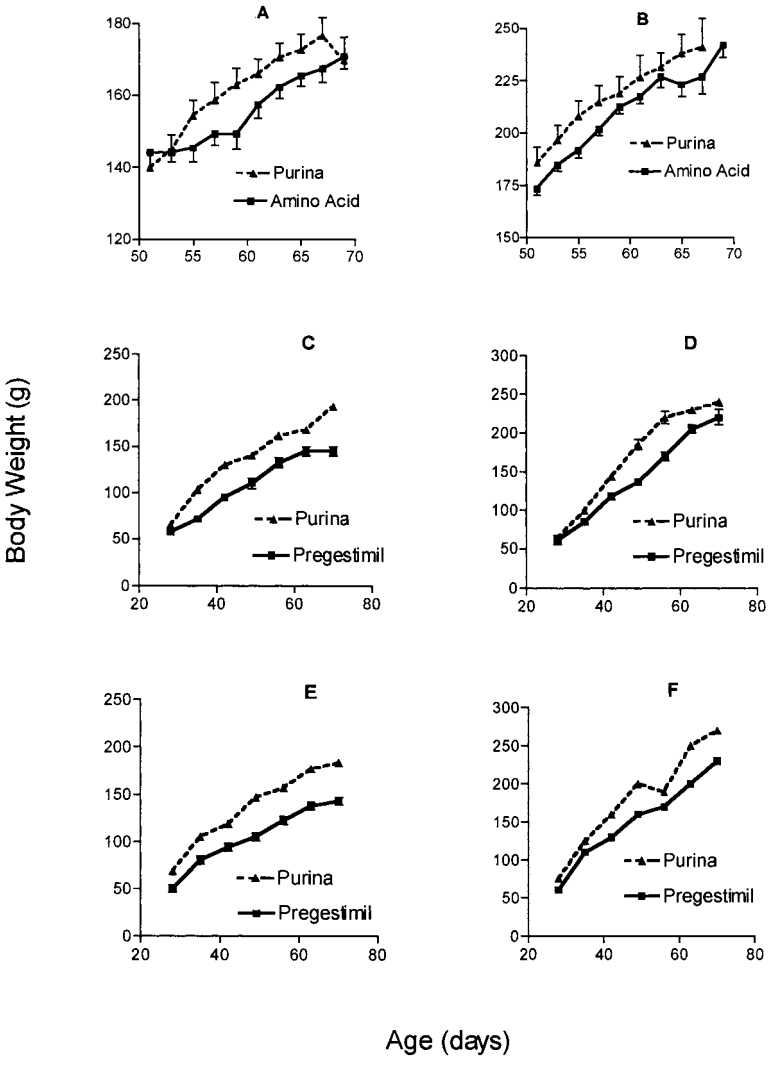

Fig 2. Weight progression by age and diet group (mean values ± 1 SEM). Rats fed a protein-free diet gained weight at a significantly lower rate than animals fed Purina chow. (A) Female F1 (DR.lyp × BB DP/Wor) rats kept at the University of Washington. (B) Male F1 (DR.lyp × BB DP/Wor) rats kept at the University of Washington. (C) Female F1 (DR.lyp × BB DP/Wor) rats kept at the University of Toronto. (D) Male F1 (DR.lyp × BB DP/Wor) rats kept at the University of Toronto. (E) Female BB DP/Wor rats kept at the University of Toronto. (F) Male BB DP/Wor rats kept at the University of Toronto.

growth curves of Purina- and protein-free diet-fed animals ($P \leq .002$ for all groups). At IDDM onset, however, amino acid-based chow and Purina chow groups at the University of Washington weighed the same (Fig 3).

Histology. No significant difference in degree of insulitis was observed between the groups of animals on the Purina rat chow and protein-free diets. Animals on the chow diet had an average score of 3.63 ± 0.65 (mean value ± SD), while

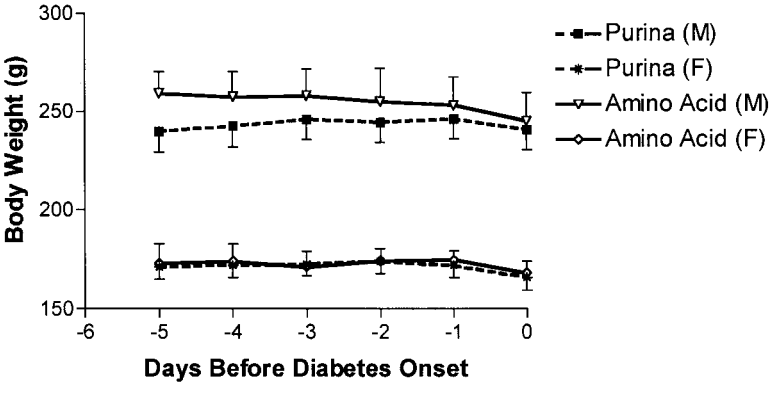

Fig 3. Weight progression by diet group immediately proceeding diabetes onset (mean values ± 1 SEM). Rats on the amino acid-based chow and Purina chow weighed the same at diabetes onset.

animals on the protein-free diets had an average score of 3.42 ± 1.06. The 2 rats remaining diabetes free in the amino acid-based diet group had moderate insulitis (score 1 and 2, respectively).

DISCUSSION

The results of this study appear to contradict the findings of many previous studies, including the first study by Elliot and Martin[10] showing that an amino acid-based protein-free diet protects BB rats from diabetes. Importantly, our findings were observed in 2 different SPF facilities, in 2 genetically related lines of BB rats, and with 2 protein-free diets that are representative of the free amino acid-based and hydrolyzed casein-based diets used previously. The slight delay in onset among F1 animals on the protein-free diets may be explained by their reduced rate of weight gain and consequent slower maturation or reduced insulin requirement due to lower food intake. It has been found that IDDM risk in BB rats increases both with the level of endogenous insulin secretion[22] and in animals with the highest body weight at 10 to 40 days of age.[23] Conversely, suppression of normal weight gain by intermittent fasting significantly reduces diabetes incidence.[24] The fact that animals from the University of Washington weighed the same in the days immediately preceding diabetes onset suggests that a certain set-point of physical maturity may be required for IDDM development, and that in some cases, animals on the test diet take longer to reach this set-point. Furthermore, no difference in degree of insulitis was observed between the groups, indicating that the onset of islet infiltration was equally rapid and severe. The 2 F1 animals remaining diabetes free on the amino acid-based diet demonstrated early to midstage infiltration by mononuclear cells, perhaps indicating a delay of, rather than protection from, diabetes.

The BB DR.lyp line was developed through the congenic introgression of the *iddm1* (*lyp*) gene onto the BB-DR background, and BB/Wor DP breeders used in the cross were the products of 70 generations of brother-sister inbreeding. Both lines are highly defined and sustain diabetes at very nearly 100% frequency when fed a standard diet and kept in SPF conditions. In contrast, most studies attributing a protective effect to a casein, hydrolyzed casein, or protein-free diet were conducted on partially outbred animals derived from the BB rat colonies maintained in Ottawa by the Canadian National Medical Research Agency. On average, 63% (range, 50% to 76% for individual studies) of these animals became diabetic when on a standard diabetogenic diet and in SPF conditions. The differential susceptibility to diabetes of the DR.lyp, DP BB/Wor, DR.lyp × DP BB/Wor, and Health Canada strains when maintained in SPF conditions and fed diabetogenic diets most likely reflects genetic heterogeneity among outbred BB rats from Ottawa and differences in the diabetogenic haplotype of inbred and outbred BB strains. One study that also used inbred DP BB/Wor animals[11] reported 48% and 54% reductions in the cumulative incidence of IDDM using 2 different hydrolyzed casein-based diets. However, it is important to note that there was only a 3% and 14% reduction in the number of animals exhibiting either IDDM or insulitis without IDDM. These results indicate that the protective effect was not as strong in BB/Wor rats as in Health Canada strains and suggest that the effect may be strain-dependent. This dependency may be a product of breeding protocols that allow some strains to retain genetic factors conferring enhanced diet sensitivity that are not present in BB/Wor or DR.lyp animals. Interestingly, the DR BB/Wor rat is not susceptible to dietary prevention of diabetes induced by poly-IC alone or in combination with $RT6^+$ cell depletion,[11] suggesting that this strain, which supplies the genetic background of the DR.lyp strain, possesses a factor conferring a resistance to protection from diabetes by dietary manipulation.

Alternatively, the reduced diabetes frequency in control DP animals in previous studies may be a function of the reduced cleanliness of the facilities. Five of the 9 studies were purportedly conducted under SPF conditions, and while control diabetes frequencies were generally higher than in studies making no mention of facility cleanliness, they are still lower than those observed in clean, inbred DP strains.[25] Infection by certain pathogens has been shown to have a significant protective effect in the BB rat,[4] and this protective effect may be amplified by the feeding of a protein-free or defined-protein diet.

In conclusion, the reduced diabetes frequency in strains that are sensitive to diet manipulation suggests a reduced robustness of these strains, in terms of diabetes susceptibility, as a cause of diet sensitivity. An antidiabetogenic diet having little or no effect on more robust (and more purely genetically determined) BB rat models may dramatically reduce diabetes incidence in a less susceptible model.

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