Short-Term Dietary Adjustment With a Hydrolyzed Casein–Based Diet Postpones Diabetes Development in the Diabetes-Prone BB Rat

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From earlier studies it appears that weaning associated changes in the animal's physiology and that of the pancreas in particular, render diabetes-prone Bio-Breeding (DP-BB) rats susceptible to the induction and development of insulin-dependent diabetes mellitus (IDDM). In this study we tested whether a short-term dietary adjustment at weaning would influence the development of diabetes later in life. For this purpose a diet in which the protein source was replaced with hydrolyzed casein (HC) was given to the rats from weaning to 60 days of age and from weaning to 130 days of age. The control group received the cereal-based standard diet throughout the experiment. The short-term dietary adjustment resulted in a significant delay of diabetes development. The rats fed the HC diet from weaning to 130 days of age showed a lower incidence of diabetes at 130 days of age. No differences were seen in the histological insulitis scores between the rats of the different treatment groups. Interestingly, when testing (mucosal) immune functions of short-term HC-fed rats, their mesenteric lymph node cells (MLNC) showed increased interferon-gamma (IFN-γ) and reduced interleukin-10 (IL-10) production after in vitro stimulation. These results demonstrate that short-term dietary adjustments at a young age can influence the course of diabetes later in life. The shift in cytokine profile of MLNC of the HC-fed rats suggests that mechanisms involved can be at the level of both the (mucosal) immune system and the β cell.

THE DIABETES PRONE Bio-Breeding (DP-BB) rat is a useful animal model for human insulin-dependent diabetes mellitus (IDDM). Like in the human situation, IDDM in the DP-BB rat develops spontaneously and is the result of the autoimmune destruction of the insulin producing β cells in the pancreas.[1] Investigations into the etiology of IDDM suggest that both genetic and environmental factors are involved.[2,3] Important environmental factors in the development of human IDDM are diet and viral infections.[2,3]

In the DP-BB rat model of diabetes, an early time interval has been identified in which diabetes can be prevented. For example, thymectomy of DP-BB rats at approximately 30 days of age prevents IDDM,[1,4] whereas thymectomy at day 60 has no effect.[4] Moreover, injection with regulatory RT6+ T cells from diabetes-resistant (DR)-BB rats in the period from 21 to 60 days of age prevents diabetes in DP-BB rats, while injection from 60 to 100 days does not influence the disease.[5] These findings indicate that the period between 21 and 60 days of age is critical for the development of diabetes in DP-BB rats.

DP-BB rats develop diabetes in a range from 60 to 120 days of age. This stage of disease development is characterized by severe insulitis.[1] The islets of Langerhans are infiltrated by macrophages, natural killer (NK) cells, and cytotoxic T cells, leading to destruction of the β cells.[1] Cytokines, particularly the proinflammatory ones such as tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1), are cytotoxic for the β cells and induce the inflammatory cascade leading to β-cell destruction.[6,7] Several reports show that increased expression in the pancreas of proinflammatory cytokines such as IL-12, TNF-α, IL-1, and interferon-gamma (IFN-γ) is associated with β-cell destructive insulitis, whereas nondestructive insulitis is more associated with increased expression of anti-inflammatory type 2 cytokines such as IL-10 and IL-4 and the type 3 cytokine transforming growth factor-beta (TGF-β).[7-9]

It is now well established that diet is an important factor influencing diabetes development in man and the animal models for IDDM: the non-obese diabetes (NOD) mouse and DP-BB rat.[10,11] Several studies have demonstrated that cereal-based diets are diabetogenic, whereas semipurified diets, where the protein source is replaced by hydrolyzed casein (HC), are protective.[11,12] The protective effect of the HC diet is dependent on timing and duration of the exposure to the diet. For example, Scott et al demonstrated that in the BB-DP rat life-long exposure to an HC diet gives the highest protection for diabetes development.[12] Reduced expression of proinflammatory cytokines in the pancreas and the induction of islet neogenesis have been mentioned as potential mechanisms for this protection.[12,13]

In view of the fact that in the DP-BB rat the period between day 21 and 60 is very important for diabetes development, we investigated whether a short-term dietary adjustment in this period can influence diabetes development later in life. For this purpose we used the HC diet as described by Scott et al[12] to reduce diabetes development in the DP-BB rat. Besides the effect on diabetes incidence and the degree of insulitis, we also studied the influence of the HC diet on the mucosal immune system by measuring pro- and anti-inflammatory cytokine profiles of isolated mesenteric lymph node cells (MLNC) and intestinal intraepithelial lymphocytes (IEL).

MATERIALS AND METHODS

Animals

DP-BB rats were kept under viral antibody-free conditions in the Central Animal Facility of the University of Groningen and received the standard cereal-based diet (Hope Farms, rodent diet no. Rmh-B2181, Woerden, The Netherlands) and acidified water ad libitum. In

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Table 1. Composition of the Control Standard Cereal-Based Diet and the Experimental HC Diet

Control Diet	HC Diet
23% Crude protein	20% Hydrolyzed casein
51% Corn starch	53% Corn starch
11.2% Sucrose	12% Sucrose
5.6% Corn oil	5% Corn oil
4.2% Cellulose fiber	5% Cellulose fiber
	Supplemental vitamin and mineral mix

this study rats from both sexes were used. In our colony, 80% to 90% of the DP-BB rats spontaneously develop diabetes before 130 days of age, with no gender differences.

All animals received humane care in compliance with the principles of laboratory animal care (NIH publication no. 85-23, revised 1985) and the Dutch law on experimental animal care.

Experimental Protocol

The modified diet, where the protein source was replaced by HC, was identical to the one developed by Scott et al.[12,13] The HC diet was a modification of the AIN-93G diet containing 20% HC (Pancase S; Redstar Bioproducts, Tara, Canada) as the source of amino acids, 53% corn starch, 12% sucrose, 5% corn oil, 5% cellulose-type fibre (Solka-Floc; Teklad, Madison, WI), supplemented with AIN-93G mineral mix and vitamin mix (ICN Biochemicals, Cleveland, OH). Table 1 shows the composition of the HC diets and the cereal-based diabetogenic control diet (Hope Farms, rodent diet no. Rmh-B2181).

DP-BB rats received the HC diet from day 21 to day 60 (short-term treatment) and from day 21 to day 130 (continuous treatment). The control group received the standard cereal-based diet throughout the study.

Rats were weighed 3 times per week and screened for hyperglycemia using blood glucose test strips (Roche diagnostics, Almere, The Netherlands). Rats were diagnosed with diabetes on the basis of a plasma glucose concentration greater than 11 mmol/L on 2 consecutive occasions. The diagnosis was confirmed by histological inspection of pancreatic tissue obtained at necropsy. Rats were anesthetized with 3% halothane in oxygen and killed by cervical dislocation.

Pancreas Histology

Upon necropsy, the pancreas was removed cleaned of fat and lymph nodes, fixed in Bouin's solution, and processed for histological analysis. Sections (7 μm) were stained with hematoxylin and eosin for evaluation of macrophage/mononuclear cell infiltration (insulitis) and degree of islet damage using a Zeiss microscope. The degree of insulitis was rated on a scale of 1 to 4 as follows: 1, normal islet appearance and no infiltration; 2, mild insulitis where macrophages/mononuclear cells are around, but not in the islets; 3, severe insulitis, where macrophages/mononuclear cells completely penetrate and infiltrate the islets; 4, end-stage islets. Per pancreas section, an average histological insulitis score was calculated by adding up the histological insulitis score of each islet and dividing it by the total number of islets counted. Per section, a minimum of 5 islets was counted. Depending on the diabetes status of the animal, the number of islets per section ranged from 5 to 20. The analysis was performed independently by 2 persons.

MLNC and IEL Isolation and Culture Procedures

Upon necropsy the mesenteric lymph nodes were removed. The lymph nodes were teased apart and passed through a 100-mesh nylon gauze. Erythrocytes were lysed with ammonium chloride buffer (0.155 mol/L NH_4CL, 0.01 mol/L $KHCO_3$, and 0.1 mol/L EDTA, pH 7.4) and the remaining cells were washed twice with RPMI (Gibco BRL, Life Technologies, Breda, Netherlands), supplemented with 10% fetal calf serum (FCS; Gibco).

IEL were isolated according to the procedure described by Todd et al.[14] MLNC and IEL were cultured in vitro in a 96-well flat-bottom culture plate (Nunc, Roskilde, Denmark), at a density of 10^5 cells per well in RPMI (Gibco) supplemented with 60 μg/mL gentamycin (Sigma, St Louis, MO), 2 mmol/L L-glutamin (Gibco), 10% FCS, and 50 μmol/L β-mercaptoethanol to a total volume of 200 μL. The cells were stimulated with 1, 2, 3, and 4 μg/mL Concanavalin A (ConA; Sigma) for 72 hours to induce cytokine production.

Cytokine Analysis

IFN-γ, IL-10, and TNF-α levels in the supernatants were measured by enzyme-linked immunosorbent assay (ELISA), using commercially available kits (OPTEIAI ELISA kits, Pharmingen, Becton Dickenson, The Netherlands) according to the manufacturer's instructions.

Statistical Analysis

The product-limit method of Kaplan and Meier was used to estimate diabetes incidence. Test groups were compared using the log-rank test. The mean insulitis scores of the groups were compared using the Mann-Whitney *U* test. We considered *P* values less than .05 to be significant. For the statistical analysis, the SPSS 8.0 software package for Windows (SPSS Inc, Chicagao, IL) was used.

RESULTS

Short-Term Dietary Adjustment With HC Postpones Diabetes Development

Continuous distribution of an HC diet to DP-BB rats has been demonstrated to protect these rats from diabetes development.[12,15] In this study we investigated whether a short-term treatment in the period early in life can also prevent diabetes development later on. For this purpose DP-BB rats received the HC diet from day 21 to day 60 (short-term treatment) and from day 21 to day 130 (continuous treatment). As demonstrated in Fig 1, continuous treatment with the HC-diet resulted in a 28% reduction of the diabetes incidence at day 130 ($P < .05$, log-rank test Kaplan-Meier survival curves) as compared to the control group, confirming the results of Scott et al.[12] However, although the 17% reduction of diabetes development was not significant, the short-term dietary adjustment postponed diabetes development as compared to the control group (Fig 1). The separate insulitis scores of the diabetic and nondiabetic rats of the different treatment groups did not differ (Table 2). However, when we calculated the total insulitis rating at kill (diabetic + nondiabetic animals), the short-term HC-treated group display a reduced insulitis score. However, it is of importance to note that this difference might be overestimated, because in the control group there is a higher frequency of diabetic animals. As expected, in all groups, the nondiabetic rats had lower insulitis scores per pancreas section compared to the diabetic rats ($P < .05$).

The protective effect of the HC diet might be the result of a reduction of proinflammatory cytokine levels during the effector phase of disease development. In that case it is likely that the mucosal immune system would be the first to be influenced by dietary adjustments. To investigate this, DP-BB rats were fed the HC diet from day 21 to day 60 and MLNC and IEL were isolated at day 60 to study their cytokine profile in vitro. As shown in Fig 2, the HC diet–fed rats had a higher IFN-γ

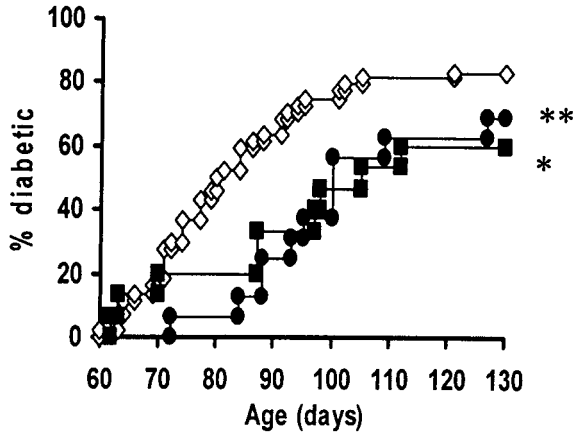

Fig 1. HC-based diets prevent and postpone diabetes development in DP-BB rats. DP-BB rats received the HC diet from day 21 to day 130 (■, continuous treatment, n = 15) and from day 21 to day 60 (●, short-term treatment, n = 15). The control rats (◇, n = 23) were fed the standard cereal-based diet used at the Central Animal Facility (RMH B2101). *Lower incidence of diabetes in continuous HC rats *v* controls, *P* < .05 log-rank test, Kaplan-Meier survival curves. **Significant delay in diabetes onset in short-term HC rats as compared to controls, *P* < .05 log-rank test, Kaplan-Meier survival curves.

production and a lower IL-10 production as compared to the rats fed the cereal-based standard diet. No differences were seen with regard to TNF-α (data not shown). Although IEL cultures showed detectable cytokine levels in vitro, no differences could be determined with regard to cytokine production between rats fed the HC diet or the standard cereal-based diet (data not shown).

DISCUSSION

The current study demonstrates that short-term dietary adjustment with HC between 21 and 60 days of age can influence the development of diabetes in the DP-BB rat. Moreover, we were able to reproduce the results of Scott et al[12] in another DP-BB rat colony. Accordingly, our results further strengthen the results of Scott et al, because in our DP-BB rat colony we had a higher incidence of diabetes compared to the colony of Scott et al (85% compared to 65%).

Previous research[12,13] has indicated that this antidiabetogenic effect of HC diets can be exerted at 2 levels: (1) the functionality of the β cell, and (2) the (mucosal) immune system. Olivares at al showed that the β cells of HC diet–fed rats produce less insulin.[16] This induced β-cell rest might reduce the level of insulitis. Janssen et al demonstrated that induction of β-cell rest by prophylactic insulin treatment reduces the infiltration of destructive macrophages into the islets of Langerhans.[17] Moreover, it was recently demonstrated that administration of an HC diet caused islet neogenesis in DP-BB rats. This islet neogenesis might be a new potential mechanism for the prevention of diabetes by the HC diet.[13]

Severe insulitis and destruction of the β cells by cytotoxic T cells, macrophages, and NK cells characterize the effector phase of diabetes development after 60 days of age in the DP-BB rat.[1] Furthermore, proinflammatory cytokines like IFN-γ, TNF-α, and IL-1, which are produced in high amount by these infiltrating cells, are cytotoxic for the β cells.[6] As was demonstrated by Scott et al, the HC diet has a strong impact on the local cytokine milieu of the pancreas in DP-BB rats.[12] The HC diet caused a reduction of the proinflammatory cytokines and an increase of the anti-inflammatory cytokines IL-10 and TGF-β, resulting in less damage to the β cell.[12]

All these mechanisms might explain why the mild insulitis (stage 1 and 2) that we observed in the nondiabetic rats of the HC diet–fed DP-BB rats frequently does not progress to the destructive form of insulitis (stage 3 and 4). Moreover, the fact that we observed changes in the cytokine profile in the MLNC cultures of HC diet–fed rats suggests the mucosal immune system also to be involved in the antidiabetogenic effect of the HC diet.

Lymphocytes in the mesenteric lymph nodes and IEL will be among the first immune cells to be affected by the diet. As shown in Fig 2, MLNC of DP-BB rats responded poorly to low levels of ConA. However, DP-BB rat leukocytes not only respond poorly to ConA, but also to other mitogens like anti-

Table 2. Histological Insulitis Score and Number of Islets per Pancreas Section of the Diabetic and Nondiabetic Rats in the Different Treatment Groups

	Diabetic		Nondiabetic (130 days of age)		Total Group	
Treatment (n)	Insulitis Score (n)	No. of Islets	Insulitis Score (n)	No. of Islets	Insulitis Score (n)	No. of Islets
Control DP-BB (23)	2.73 ± 0.68 (19)	6.75 ± 2.75	1.41 ± 0.68 (4)	12.00 ± 4.95	2.41 ± 0.86 (23)	8.07 ± 3.87
HC day 21-60 (16)	2.13 ± 0.02 (11)	10.62 ± 6.90	1.13 ± 0.10 (5)	9.25 ± 2.30	1.52 ± 0.50* (16)	10.19 ± 5.43
HC day 21-130 (15)	ND	ND	1.51 ± 0.30 (6)	12.87 ± 7.11	—	—
			Nondiabetic (56 days of age)			
56-day-old DP-BB (3)	—	—	1.02 ± 0.03 (3)	18.33 ± 1.53	—	—

NOTE. The results are expressed as the mean histological insulitis score ± SD.

Abbreviation: ND, not determined.

*$P < .05$ (Mann-Whitney U test) compared to control DP-BB rats.

Control DP-BB and 56-day, old DP-BB rats were fed the standard cereal-based diet of Hope Farms (RMH-B2101). The HC groups were fed the HC-diet for the periods as indicated.

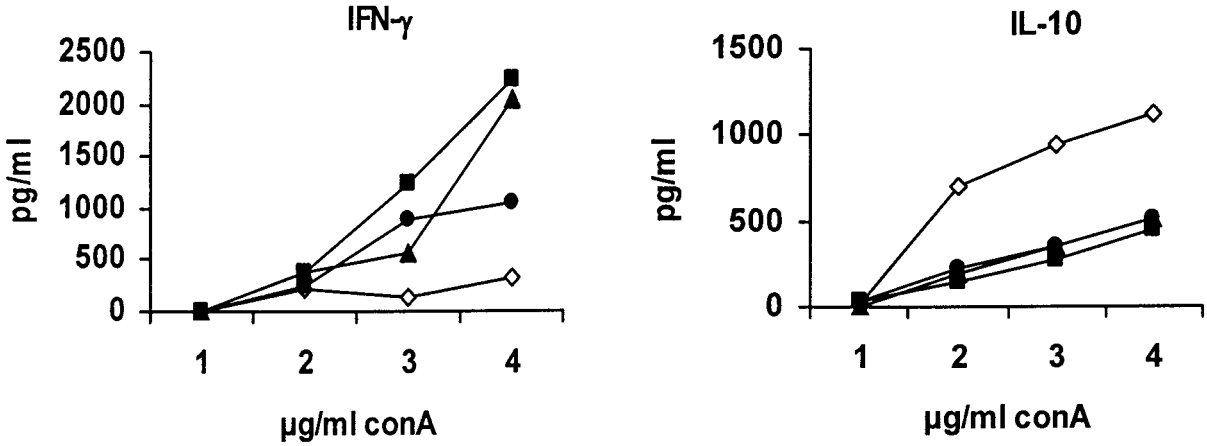

Fig 2. MLNC of HC-diet fed rats produce more IFN-γ and less IL-10 in vitro. DP-BB rats received the HC-diet from day 21 to day 60 of age. At day 60 the rats were killed, their MLNC isolated, and put in culture as described in the Methods. Control rats were fed the standard diet used at the Central Animal facility. Three individual rats receiving the HC diet (■, ▲, ●) and 1 control rat (◇) are shown. The figure is a representative of 2 separate experiments.

CD3 and lipopolysaccharide (LPS).[18,19] In the case of T cells, this is caused by nitric oxide production of macrophages.[19] When these macrophages were depleted the T cells responded quite well to ConA with regard to proliferation.[19] Moreover, the lymphopenia gene–induced limited life span and proliferative capacity of DP-BB rat T cells will negatively affect in vitro T-cell responsiveness of such cells. We also have indications that the loss of the regulatory RT6-positive T cells in DP-BB rats makes the monocytes less responsive to LPS with regard to IL-10 and TNF-α production.[18] When DR-BB rats were depleted of their RT6-positive T cells, the cytokine production of their monocytes dropped to levels comparable with DP-BB rats.[18]

Interestingly, we see a change in the cytokine profile of the MLNC, but not in IEL cultures induced by the HC diets. This is probably due to the fact that in mesenteric lymph nodes there are interactions between T cells and antigen-presenting cells draining from the gut.[20] These interactions will lead to activation and differentiation of T cells. In the IEL we did measure cytokine production, but no change in the cytokine profile of the animals receiving the HC diet. This suggests that activation and subsequent cytokine polarization might predominantly occur in the mesenteric lymph node and less in the epithelial layers of the gut.

Surprisingly, we observed an increased IFN-γ and a reduced IL-10 production by the MLNC cultures in vitro. This is in contrast with the findings of Scott et al,[12] who demonstrated reduced IFN-γ and increased IL-10 levels in the islets of Langerhans at the mRNA level. This is possibly related to the different sources of T cells used in these 2 studies: MLNC versus pancreas-infiltrating leukocytes. The interaction between antigen-presenting cells and T cells in the mesenteric lymph nodes and the lack of such an activating microenvironment in the pancreas might explain the observed differences. The 2 populations may also represent different T-cell subsets or T cells at different stages of differentiation. Nevertheless, the induction of high IFN-γ responsiveness by the HC diet in MLNC could well be beneficial, since it has been demonstrated that IFN-γ prevents diabetes development in DP-BB rats when given lifelong immediately after weaning.[21,22] This protective effect of IFN-γ is probably caused by the induction of regulatory T cells.[21,22]

Feeding the HC diet to DP-BB rats obviously also prevented or delayed the introduction of potential diabetogenic food antigens in the regular diet. Cow's milk proteins, such as casein, lactoglobulin, albumin, and (pro)insulin, have been suggested to trigger disease.[10,23] However, it has become clear that also other food ingredients such as wheat gluten and soy bean proteins,[11] but also bacterial antigens (eg, hsp60), can influence the development of diabetes.[24] The mode of action of such diabetogenic antigens is supposed to be via crossreactivity (mimicry) with β-cell antigens. Indeed, homologies have been described between, for example, β-casein and p69 carboxypeptidase and GLUT2,[25] and between NADH ubiquinone reductase in wheat and soy bean and tyrosine phosphatase IA2.[26]

Diabetes development in man and the DP-BB rat is multifactorial. The immune system, the β-cell functionality, viral infections, and diabetogenic food antigens are the key players in diabetes development. This multifactoriality can explain why the timing and duration of the HC diet treatment has differential effects on the development of diabetes in the DP-BB rat. Finally, our results show that with regard to humans, short-term treatment with an HC diet has therapeutic potential.

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