Potential Mechanisms by Which Certain Foods Promote or Inhibit the Development of Spontaneous Diabetes in BB Rats

Dose, Timing, Early Effect on Islet Area, and Switch in Infiltrate From Th1 to Th2 Cells

Fraser W. Scott, Heather E. Cloutier, Rainer Kleemann, Ute Wöerz-Pagenstert, Paul Rowsell, H. Wayne Modler, and Hubert Kolb

Certain diets can have major effects on the development of IDDM in DP-BB rats, but data are scant on the timing, dose, and mechanisms involved. We therefore determined the dose response, timing, and duration of exposure required to induce diabetes, and characterized the effects of nutritionally adequate diets with widely different diabetogenicity on the pancreatic islet area and cytokines. DP-BB rats were fed a diabetogenic, cereal-based, NIH-07 (NIH) diet or a protective, casein or hydrolyzed casein (HC)-based, semipurified diet. Rats were fed from weaning to 50 or 100 days with the HC diet and then switched to the NIH diet, or fed the NIH diet from weaning to 50 days and switched to the HC diet. Pancreas histology and diabetes outcome were determined. Semiquantitative morphometric analyses of hematoxylin and eosin-stained sections of pancreas from 41-day-old rats were also carried out. Diet-induced effects on pancreatic cytokine levels were measured at 70 days using reverse transcriptase–polymerase chain reaction analysis of γ-interferon (IFN-γ), interleukin-10 (IL-10), and transforming growth factor-β (TGF-β). Long-term daily exposure, particularly around the beginning of puberty to late adolescence (50–100 days), was important for development of diabetes. DP-BB rats could be rescued from diabetes development by feeding them a low-diabetogen HC diet as late as 50 days. Diabetes frequency was highest in rats fed 70% and 100% NIH diets. By age 41 days, before classic insulitis, the islet area in HC-fed DP-BB rats was 65% greater than in NIH-fed rats. By 70 days, when mononuclear cells were visible in the islets of most NIH-fed, but not HC-fed rats, the more pronounced inflammatory process in NIH-fed rats was associated with a Th1 cytokine pattern (high IFN-γ and low IL-10 and TGF-β), whereas the pancreases of HC-fed rats showed fewer infiltrating cells, low levels of IFN-γ, and high levels of TGF-β, typical of a Th2 cytokine pattern. Thus dietary modification can occur as late as puberty. Further, long-term exposure to sufficient amounts of food diabetogens between 50 and 100 days was required for maximum diabetes induction. The islet area was modified by diet before signs of classic insulitis. Pancreatic inflammation in NIH-fed animals is a Th1-dependent phenomenon. The HC diet inhibited insulitis and was associated with a Th2 cytokine pattern in the pancreas, protecting diabetes-prone rats from developing diabetes. Diabetes 46:589–598, 1997

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he role of diet in the development of IDDM has been enigmatic and controversial because of 1) difficulties in obtaining accurate, long-term food intake data from people with genetic risk for IDDM destined to become diabetic, and 2) incomplete understanding of the effects of defined diets on the major animal models of spontaneous, autoimmune diabetes. Studies in humans (1,2) have focused almost exclusively on the period of early infancy and the possible role of breast-feeding and/or exposure to a single diet component, cow milk protein, using retrospective data obtained through case-control studies (3) and ecological analyses (4). The controversy whether exposure to cow milk in infancy is the triggering event for the development of diabetes (5–10) emphasizes not only the difficulties involved in interpreting retrospective infant data (3), but also might indicate involvement of other diet components and different critical exposure times.

We have reported that spontaneous diabetes in the DP-BB rat is largely food dependent and that incidence is highest in animals fed mainly plant-based diet mixtures (11–14). Characterizing the major components of standard rodent diets demonstrated that most of the diabetogenic potential resides in wheat (15) and soy (16) protein components (see APPENDIX). Although skim milk powder–based diets sometimes produced a higher incidence of diabetes compared with low-dia-
betagen, negative-control diets, this difference was not consistent or statistically significant \( (P = 0.14) \) (13,14). When DP-BB rats are fed nondiabeticogenic amino acid sources, such as casein, hydrolyzed casein (HC), lactalbumin, hydrolyzed lactalbumin, hydrolyzed soy protein, fish meal, and other sources that apparently lack or have reduced diabetes-inducing potential, \( \geq 80\% \) of the animals remain clinically diabetes-free well into adulthood. These diets also inhibit the inflammatory process in the islets and delay onset of diabetes in the few animals that go on to develop clinical symptoms (13,14). However, details of the mechanisms of this dietary control of pathogenesis are not clear.

The phenotype of \( \beta \)-cells is normally low major histocompatibility complex (MHC) class I expression, with no detectable class II expression in in situ islets from newly diagnosed human patients, DP-BB rats (17,18), and NOD mice (19). Macrophages are the first cells to infiltrate the islets (17), and when they are inhibited by treatment with silica, diabetes is prevented (20,21). A recent report (22) showed that feeding a diabetogenic, plant-based diet caused hyperexpression of MHC class I molecules on \( \beta \)-cells as early as age 24 days, about 10 days after the pups begin to nibble on the dam’s solid food. This hyperexpression was apparent even in silica-treated, macrophage-inhibited DP-BB rats. This suggested that the feeding of a diabetogenic diet enhanced \( \beta \)-cell antigenicity early in life through a non-macrophage-dependent process, and that the early phase of the diet-diabetes interaction was focused on the target cells.

When DP-BB rats are about age 60 days, flow cytometric analyses show that feeding a protective HC diet from weaning is associated with a small but statistically significant \( (P < 0.05) \) decrease in the percent of macrophages in peripheral blood (23). Similar results were recently reported in spleen and mesenteric lymph nodes (24). However, these changes in immune cell phenotype distribution did not correct the immune abnormalities in BB rats. Also, selected measures of immune cell activity show only minor diet-induced alterations that cannot explain the dramatic protection seen in DP-BB rats fed an HC diet.

Because diet-induced changes in target cell expression of MHC class I antigens occur at a very early age (18,22), before classic insulitides, it may be assumed that certain diabetogenic diets act through an early effect on the target cells. This early interaction may not be sufficient by itself to result in expression of diabetes, as this outcome may require long-term exposure to food diabetogens in sufficient amounts to cause changes in the islet tissues that attract the infiltrating leukocytes that damage \( \beta \)-cells.

To test this hypothesis, we used diets with similar nutritional and growth-promoting properties (25) but widely different, well-characterized diabetogenicity (13–16). The protective diet was a modified American Institute of Nutrition semipurified diet (AIN-76A) with casein or HC as the amino acid source; the diabetogenic diet (13,16) was a mainly plant-based, standard diet with specified amounts of ingredients originally developed at the National Institutes of Health, called NIH-07 (NIH) (25). NIH and HC diets were fed to DP-BB rats to define dose, timing, and duration of exposures required to induce diabetes, as well as changes in islet tissue area and cytokine patterns of islet-infiltrating lymphoid cells.

Our results showed that 1) the diabetogenicity of the NIH diet can be diluted in a dose-dependent manner, 2) exposure to food diabetogens is particularly important between puberty and late adolescence, and 3) feeding DP-BB rats a low-diabetogenic HC diet maintained islet area, inhibited subsequent leukocyte infiltration, and changed the cytokine pattern transcribed in the pancreas from Th1 to Th2.

**RESEARCH DESIGN AND METHODS**

**BB rats.** B6c (controls) and DP-BB rats were obtained from the Animal Resources Division of Health Canada where they were maintained under viral antibody-free conditions. The colony is antibody-free with respect to Sendai virus, pneumonia virus of mice, rat coronaviruses/sidalacryoadenoviruses, Kilham rat virus, Toxocara H-1 virus, reovirus type 3, and mycoplasma pulmonis. Animals were maintained in banks of 30 stainless steel, wire-bottom cages, and were permitted free access to food and water. After age 60 days, they were tested twice weekly for glucose in the urine (Tetapaste; Lily, Toronto, Ontario). Those rats with a value of 22 were fasted overnight, and blood glucose was measured in the morning with a blood glucose meter. Animals with blood glucose >200 mg/dl (11.1 mmol/l) were killed by exsanguination under 2% isoflurane in oxygen anesthesia.

**Diet.** Animals were weaned at day 23 onto one of three diets: 1) NIH-07, a standard, open formula, cereal-based diet fed in meal form; 2) powdered semipurified casein-based diet; or 3) an HC-based semipurified diet (the latter two diets were modifications of the AIN-76A recommended diet for rats and mice) (16). For the dose-response experiment, the HC and NIH diets were mixed as described below. The NIH diet was purchased from Zeigler Brothers (Gardner, PA) and had the following composition: dried skim milk (5%), fish meal (10%), HYANN drinks (12%), alfalfa meal (4%), corn gluten meal (3%), ground corn (24.5%), ground hard winter wheat (23%), wheat middlings (10%), Brewer's yeast (2%), molasses (1.5%), soybean oil (2.5%), and minerals and vitamins (see 25 for details). The semipurified diets contained either 50% casein (vitamin-free; Teklad, Madison, WI) or 20% HC (Pancas; Chaplin Industries, Mississauga, Ontario) as the source of amino acids. Both semipurified diets also contained the following: 52% corn starch, 12% sucrose, 5% corn oil, 5% cellulose-type fiber (Solka-Floc; Teklad), supplemented with AIN-76 mineral mix and AIN-76A vitamin mix (ICN Biochemicals, Cleveland, OH). We have confirmed that a casein-based AIN-50 diet (26), in which minor nutrient modifications are recommended compared with our previously modified AIN-76A diets, was also protective, resulting in a diabetes incidence of 11% by 156 days.

**Dose-response experiment.** To determine if the diabetogenic activity of the NIH diet could be diluted, we fed four groups of DP-BB rats \( (n = 4–15/group) \) from weaning to 133 days the following diets: 1) a protective HC-based diet, a diabetogenic NIH diet, or one of two diet mixtures in which NIH:HC ratios were either 3:7 or 1:1. Diabetic animals were killed by exsanguination within 24 h of diagnosis, and remaining rats were killed around 133 days. Pancreatic sections were prepared for determining insulitis score as described below.

**Time of introduction and duration of exposure.** To determine the effect of time of introduction and duration of exposure to diabetogenic diet, the following study was carried out. Following weaning, DP-BB rats were fed an HC diet (negative control) or an NIH diet (positive control); at 50 days the diets were switched, HC→NIH and NIH→HC. To see if late exposure to food diabetogens would lead to diabetes, animals were fed with HC until 100 days and then switched to NIH. Diabetic animals were killed within 24 h of diagnosis, and those that remained asymptomatic at 186 days were killed by exsanguination under anesthesia, and pancreas histology and diabetes incidence were compared.

**Pancreas histology.** Pancreases were removed, cleaned of fat and lymph nodes, and fixed in Bouin’s solution. Sections (5 mm) were cut at 100-μm intervals and stained with hematoxylin and eosin for evaluation of macrophage/mononuclear cell infiltration (insulitis) and degree of islet damage using a Zeiss Photomicroscope III (Carl Zeiss, Don Mills, Ontario, Canada) equipped with a 2.5 X objective and 12.5 X ocular with rings for diameter measurement. All islets in two separate sections at least 100 μm apart were evaluated. Depending on the diabetes status of the animal, numbers ranged from 5–36 islets/section. Briefly, a subjective rating was given for the extent of insulitis graded on a scale of 1 to 5 (16), as follows: no insulitis, no infiltrate (0); mild insulitis (1) with no inflammation; 2, mild insulitis with a similar number of islets but about 30% infiltrated; 3, a majority of islets (80%) infiltrated; 4, fewer islets remaining (half), with most infiltrated or very small; 5, end-stage disease, with usually no more than 5 very small islets, with little inflammatory infiltrate remaining.

**Semiquantitative morphometric analysis.** This analysis was carried out as follows: pancreas sections were scanned to locate all of the islets. To define islet size, circular rings of 0.63, 1.6, and 4.0 were used with areas of 0.02, 0.17, and 0.96 mm² using a 2.5 X objective. Islets were classified using the following average areas: small islets were
A graph summarizing the results of several experiments in Fig. 1. These data show that feeding an NIH diet produced far more cases of diabetes than feeding casein or HC diets.

RESULTS

Cytokine assay. To determine if diet could affect cytokine levels in the pancreas, animals were weaned onto an HC or NIH diet and then killed by exsanguination while under isoflurane anesthesia at age 70 days. Two other groups of animals were treated by switching diets at 50 days, as in the timing study. At age 70 days, the pancreas was removed, cleaned of fat and lymph nodes, and split longitudinally in half. Total RNA was isolated from the fresh, snap-frozen pancreatic tissue using acid guanidinium thiocyanate-phenol-chloroform extraction. Synthesis of cDNA was carried out under RNase-free conditions using 6 μg of each RNA sample with a Superscript Reverse Transcriptase kit (Gibco BRL, Eggenstein, Germany). Polymerase chain reaction (PCR) amplification was carried out in the same reaction tube under mineral oil after adding a Taq Polymerase kit (USB/Amersham, Buchler, Braunschweig, Germany). Specific primers for γ-interferon (IFN-γ), transforming growth factor β (TGF-β) (Clontech Laboratories, Palo Alto, CA), interleukin-10 (IL-10) [27], and β-actin (3′ primer TCATAAGATGGGCCAGACTGTG, 5′ primer TGAAGGCCAACCCTGAGAAG) were used in both steps of reverse-transcriptase (RT)-PCR. Amplified products were separated by electrophoresis on a 2% agarose gel. Control experiments to check the reproducibility of results and quantitation of PCR products were carried out as described previously [28] by hybridization to a specific 32P-labeled probe and determining 32P-stimulated luminescence by phosphorimaging. Counts obtained for cytokine mRNA were calibrated to the amount of mRNA detected for a constitutively expressed housekeeping gene, β-actin, which was set equal to 1.

RESULTS

A graph summarizing the results of several experiments in which we fed three diets used as positive control, NIH diet, or negative controls, casein or HC diets, on the number of BB rats that developed diabetes and the insulin score is shown in Fig. 1. These data show that feeding an NIH diet produced far more cases of diabetes than feeding casein or HC diets. The peak incidence in NIH-fed rats occurred around 91 days, whereas that for HC and casein-fed rats occurred at about 108 days. In all three groups, after the peak incidence was reached, the number of rats that developed diabetes decreased and remained at low levels up to ages 180–210 days. There was no indication of a later trend showing an increase in the number of rats becoming diabetic, regardless of diet. In DP-BB rats fed the NIH diet, the mean insulin score in all rats or in rats remaining asymptomatic at the end of the experiment was significantly higher than the mean scores from casein- or HC-fed rats (P = 0.00001).

The dose-response results are shown in Fig. 2. Diabetes incidence and the mean insulin scores were low in HC-fed rats (0% NIH in the diet) and highest in animals fed 70% and 100% NIH diets. At the higher dilution of 3 parts NIH to 7 parts HC, diabetes incidence and insulin scores were similar to those seen when DP-BB rats were fed the HC diet alone. Insulin score was highest in the DP-BB rats fed 100% NIH diet, lowest in rats fed 0% or 30% NIH, and moderately high in rats fed the 70% NIH diet. Beginning at 30% NIH in the diet, final diabetes incidence increased as the amount of NIH in the diet increased (Fig. 2C). Final body weight was similar in asymptomatic rats remaining at the end of the experiment, regardless of diet.

Results of the timing study are shown in Fig. 3. Diabetes frequency and insulin scores and frequency were lowest in HC-fed rats. Delaying exposure of DP-BB rats to the NIH diet from weaning at 23 days until 50 days delayed onset by the same amount of time (i.e., 28 days, P = 0.02; Table 1). These rats ultimately became diabetic at the same rate and with a diabetes incidence indistinguishable from those exposed at weaning and maintained exclusively on the NIH diet. Animals that were fed a protective HC diet until 100 days were less likely to develop diabetes than animals exposed to food diabetogens throughout the experiment (NIH group) or from 50 days to the end. Animals that were fed the NIH diet from weaning but switched to an HC diet at 50 days had a lower rate of disease appearance and diabetes frequency; values for insulin grade and frequency were similar to those of rats fed the protective HC diet. Those animals that did develop diabetes showed a similar age of onset to NIH-fed rats. In animals that remained asymptomatic at the end of the study, 90% of those first exposed to the NIH diet at 50 days had higher insulin scores (Table 1) compared with those exposed to NIH only at 100 days or switched from NIH to HC at 50 days. Overall, these results suggested that long-term, constant
exposure to diabetogens in the diet was particularly important around puberty.

To determine if diet might affect the target tissue itself before macrophages and mononuclear cells are generally visible in the islets, we examined the effect of feeding either the HC or NIH diet to DP-BB rats from weaning until age 41 ± 4 days. The results of the semiquantitative morphometric analyses of the pancreas are shown in Table 2. The total islet area in HC-fed DP-BB rats was 65% greater than in NIH-fed DP-BB rats (0.60 ± 0.26 vs. 0.36 ± 0.16 mm²; *P* = 0.05). This was primarily attributable to a decrease in the number and total area of medium-size islets. The HC diet maintained the same islet area in DP-BB rat pancreas as that observed in BBc rats. Islet area in BBc rats was not affected by diet (data not shown). The proportion of the pancreas that was islet tissue was greater in HC-fed rats compared with NIH-fed DP-BB rats (1.1 ± 0.33 vs. 0.64 ± 0.23%; *P* = 0.006) and BBc rats regardless of diet.

To examine the possibility that a diet low in diabetogenic activity might have changed the cytokine levels expressed in pancreas, we measured IFN-γ, IL-10, and TGF-β mRNA in pancreas RNA from DP-BB animals fed the NIH diet or the protective HC diet from weaning. The analysis was carried out at age 70 days, which is when NIH-fed BB rats start to develop IDDM (Figs. 1-3). The results of the cytokine analyses are shown in Fig. 4. IFN-γ was significantly higher in NIH-fed rats at 70 days compared with HC-fed rats of the same age (*P* = 0.0001). TGF-β levels were markedly higher in HC-fed DP-BB rat pancreases (*P* = 0.003), and IL-10 values tended to be higher in HC-fed rats, but this difference was not significant. Thus the cytokine profiles in the pancreas of HC-fed rats, compared with NIH-fed and BBc rats, showed consistent differences that were related to the diabetogenic activity in the diet.

FIG. 2. Dose response to a diabetogenic NIH diet mixture fed to DP-BB rats. Diets were mixed in the proportions shown, and fed to DP-BB rats from weaning to 140 days (n = 14-15/group). The effects of increasing the ratio of NIH:HC on 1) diabetes outcome (panel A; survival), 2) islet inflammation in all rats and in those that remained asymptomatic at the end of the study (panel B; insulitis score), and 3) final diabetes incidence (panel C) are shown. For details of animals, diets, and scoring of insulitis, refer to METHODS. HC, hydrolyzed casein-based (protective) semipurified diet; NIH, an open-formula diet that is mainly (83%) plant-based and that has been shown to be diabetogenic in DP-BB rats.

FIG. 3. Timing of the diet-diabetes interaction. DP-BB rats were fed from weaning an HC-based diet alone or NIH diet alone as negative and positive controls, respectively. One group of rats was fed the HC diet from day 23 to day 50 and then switched to NIH (A). Another group was fed HC from weaning to age 100 days and then switched to NIH (B). A third group was fed NIH from day 23 to day 50 and then switched to HC (C). The mean insulitis score is plotted in the inset of each panel; numbers on the survival curves match the numbers in the bar graphs. There were 27 rats in all groups except the NIH-fed group, which had 26.
TABLE 1
Switching or delaying exposure to the diabetogenic NIH diet in DP-BB rats

<table>
<thead>
<tr>
<th>Diet</th>
<th>Diabetes</th>
<th>P</th>
<th>Onset</th>
<th>P</th>
<th>Insulitis*</th>
<th>P</th>
<th>Insulitis score</th>
<th>P</th>
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</thead>
<tbody>
<tr>
<td>HC</td>
<td>7/27 (26)</td>
<td>0.006 vs. NIH</td>
<td>129 ± 21</td>
<td>0.09 vs. NIH</td>
<td>13/27 (48)</td>
<td>0.05 vs. NIH</td>
<td>2.0 ± 1.3</td>
<td>0.0004 vs. NIH</td>
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<td>All rats</td>
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<td>HC→NIH, 50 days</td>
<td>17/27 (63)</td>
<td>0.008 vs. HC</td>
<td>132 ± 35</td>
<td>0.88 vs. HC</td>
<td>26/27 (96)</td>
<td>0.0001 vs. HC</td>
<td>3.7 ± 1.2</td>
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<tr>
<td>HC→NIH, 100 days</td>
<td>9/27 (33)</td>
<td>0.78 vs. HC</td>
<td>135 ± 39</td>
<td>0.72 vs. HC</td>
<td>19/27 (70)</td>
<td>0.11 vs. HC</td>
<td>2.8 ± 1.5</td>
<td>0.03 vs. HC</td>
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<td>NIH→HC, 50 days</td>
<td>8/27 (30)</td>
<td>1.0 vs. HC</td>
<td>105 ± 33</td>
<td>0.17 vs. HC</td>
<td>16/27 (59)</td>
<td>0.43 vs. HC</td>
<td>2.4 ± 1.6</td>
<td>0.25 vs. HC</td>
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Data are means ± SD or n (%) unless otherwise indicated. Rats were fed the indicated diets from weaning either throughout the experiment or were switched as indicated. Animals that became diabetic were killed within 24 h of diagnosis. The remaining animals were killed at ~185 days. Insulitis was scored as described in METHODS. Analysis was carried out using one-way ANOVA with least significant difference test or Fisher’s exact test (two-tailed). *≥2 insulitis score (see METHODS for details); values are given for all rats and rats remaining nondiabetic at 185 days.

fed animals were characteristic of Th2 cells, with IFN-γ low, TGF-β high, and a tendency for IL-10 levels to be increased. By contrast, the NIH-fed animals had high IFN-γ and low IL-10 and TGF-β. Hence the HC diet appeared to have an impact on cytokine gene expression in the pancreas as well as target tissue homeostasis.

Because the timing experiment (Fig. 3) showed that feeding the NIH diet up to day 50 and then switching to an HC diet (NIH/HC) could still be protective compared with delaying exposure to NIH to day 50 (HC/NIH), cytokine messages for IFN-γ, IL-10, and TGF-β were measured at day 70 in these two separate groups of DP-BB rats. IFN-γ levels were similar in both groups, but TGF-β levels tended to be higher in the (protected) NIH/HC group (Fig. 5). Also, TGF-β levels in the NIH/HC group were not different from rats fed HC exclusively (Fig. 4). This was not the case in the HC/NIH group, in which TGF-β levels were significantly decreased compared with HC-fed rats (P = 0.04). In the protected NIH/HC group, the IL-10 levels tended to be higher than the HC/NIH group. More importantly, the NIH/HC group had higher levels of IL-10 than those rats fed NIH exclusively (P = 0.05). Although there was not a clear demarcation between the two diet-switch treatment groups, as was seen when comparing HC and NIH (Fig. 4), there were some differences, suggesting that even as late as 50 days removal of dietary diabetogens caused a favorable alteration in pancreatic cytokine levels.

DISCUSSION
There is now a considerable body of evidence that spontaneous diabetes in DP-BB rats and NOD mice is to a large extent food related. Since the first experimental evidence was reported more than a decade ago (11,12,29), the dramatic effect that various dietary protein sources have on expression of diabetes in DP-BB rats has been confirmed repeatedly, by us (11-16,22,30-32) and other groups (18,33-36). Dietary components also control development
of diabetes in NOD mice (30,37-43). The data in Fig. 1 show that diet has a major impact on the appearance of overt diabetes and pancreatic inflammation. The fact that a later peak of diabetes incidence was not observed in rats fed casein or HC diets suggests, but does not prove, that feeding a low-dia-
betogenic diet prevented a majority of DP-BB rats from devel-
oping IDDM. The data in Fig. 1 show that most of the diabetes
in our BB rats is diet-related, that is:

\[
\text{NIH diabetes incidence - HC diabetes incidence} = \frac{62 - 12}{62} = 81\% \text{ food-related cases}
\]

This is further illustrated by the data in Figs. 2 and 3.

The source of dietary amino acids is an important factor con-
trolling diabetes expression. Indeed, certain dietary amino acid sources are not diabetogenic, whereas others produce intermediate or high diabetes incidence (13,14). However, diabetes apparently does not depend on a defi-
ciency of dietary amino acids, as it can still be induced in animals fed a (diabetogenic) wheat gluten diet supple-
mented with amino acids (15). Standard laboratory diets for rodents, which are mostly plant based, produce the highest diabetes incidence in BB rats and NOD mice. The diabeto-
genicity of each of the major components of the standard, cereal-based NIH diet has been determined (13); the major food diabetogens affecting the DP-BB rat are wheat (15), soy (16), and, to a variable degree, cow milk protein sources (10,13,29). Wheat and soy are also reported to be diabetogenic when fed to NOD mice (41, see also ref. 31). There are reports that skim milk powder diets are not diab-
etogenic in NOD mice (37), whereas the major milk pro-
tein, casein, has been found to be protective in some NOD
lines (37,40) but is diabetogenic in others (39). These data suggest that, as in the BB rat, cow milk protein sources are variably diabetogenic in NOD mice. Nonetheless, there is

remarkable agreement among studies using both NOD
mice and DP-BB rats that certain nondiabetogenic amino acid sources, particularly HC-based diets, are protective (13,16,22,30,32,38,41,43) and that wheat and soy are dia-
etogenic (13,15,16,31,41).

It should be noted that the generally accepted terms dia-
etogenic versus protective diet imply knowledge of
the underlying mechanism, which is presently not available. In principle, a diabetogenic diet may trigger or promote auto-
immune reactivity, or it may be neutral to the sponta-
neously developing disease process. Similarly, a protective diet may be lacking diabetes-inducing constituents, or it may
actively induce a protective response against diabetes. To
address this issue, we performed mixing experiments with
protective (HC) and diabetogenic (NIH) diets. The results
reported here do not indicate the induction of a diabetes-sup-
pressing activity by the HC diet. Rather, there is a dose
response to the amount of NIH in the diet that is associated
with a concomitant change in islet inflammation. This is in
keeping with the identification of several nondiabetogenic
protein sources (13) and the finding that, when concentrated
sources of wheat and soy proteins (the major components of
the NIH diet) are fed as the sole source of dietary amino acid sources instead of or in addition to HC in semipurified diets, they are
diabetogenic. It is possible to decrease the diabetogenicity
of the NIH diet by various chemical treatments (14). This further
suggests that it is the presence of diabetogenic compounds
in the NIH diet that is more important than a potential pro-
tective function attributable to HC (or other nondiabeto-
genic amino acid sources).

TABLE 2

<table>
<thead>
<tr>
<th>Diet-related changes in pancreatic islets of young DP-BB rats</th>
<th>HC</th>
<th>NIH</th>
<th>P*</th>
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</thead>
<tbody>
<tr>
<td>n</td>
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<td>0.84</td>
</tr>
<tr>
<td>Body wt (g)</td>
<td>87 ± 10</td>
<td>88 ± 7</td>
<td>0.09</td>
</tr>
<tr>
<td>Pancreas wt (g)</td>
<td>0.23 ± 0.04</td>
<td>0.26 ± 0.04</td>
<td>0.09</td>
</tr>
<tr>
<td>Pancreas/body wt × 10^3</td>
<td>2.6 ± 0.4</td>
<td>3.2 ± 0.8</td>
<td>0.09</td>
</tr>
<tr>
<td>No. small islets†</td>
<td>20 ± 7</td>
<td>16 ± 8</td>
<td>0.38</td>
</tr>
<tr>
<td>No. medium islets†</td>
<td>5 ± 3</td>
<td>3 ± 2</td>
<td>0.03</td>
</tr>
<tr>
<td>Total no. islets</td>
<td>25 ± 9</td>
<td>19 ± 8</td>
<td>0.20</td>
</tr>
<tr>
<td>Small islets, total area (mm^2)</td>
<td>0.20 ± 0.07</td>
<td>0.16 ± 0.08</td>
<td>0.38</td>
</tr>
<tr>
<td>Medium islets, total area (mm^2)</td>
<td>0.40 ± 0.21</td>
<td>0.20 ± 0.12</td>
<td>0.03</td>
</tr>
<tr>
<td>Total islet area (mm^2)</td>
<td>0.60 ± 0.26</td>
<td>0.36 ± 0.16</td>
<td>0.05</td>
</tr>
<tr>
<td>As % pancreas</td>
<td>1.1 ± 0.3</td>
<td>0.6 ± 0.2</td>
<td>0.006</td>
</tr>
<tr>
<td>Subjective histology rating†</td>
<td>1.1 ± 0.2</td>
<td>1.0 ± 0.0</td>
<td>0.33</td>
</tr>
</tbody>
</table>

Data are means ± SD. DP-BB rats were ages 41 ± 4 days. *Student's t test for independent variables. †See METHODS for more information.

FIG. 4. Cytokine levels in pancreas mRNA isolated from DP-BB rats fed an HC-based diet or NIH diet. Cytokines (IFN-γ, IL-10, and TGF-β) were measured using RT-PCR analysis of mRNA prepared from pan-
creases of DP-BB rats fed either a diabetogenic NIH diet or a protec-
tive HC diet from weaning to the age at kill (70 days). Each point rep-
resents one animal. The values shown are mRNA levels determined by
RT-PCR, followed by quantitation of radiolabel by phosphor-stimulated
luminescence and calibrated to the amount of β-actin mRNA (set as 1).
Bars indicate means ± 1 SD.
FIG. 5. Cytokine levels in pancreas mRNA isolated from DP-BB rats fed HC-based or NIH diets to 50 days, when diets were switched. Cytokines (IFN-γ, IL-10, and TGF-β) were measured at age 70 days using RT-PCR analysis of mRNA prepared from pancreases of DP-BB rats fed either a diabetogenic NIH diet or a protective HC diet from weaning to 50 days. At 50 days, the diets were switched NIH→HC and HC→NIH. Each point represents one animal. The values shown are mRNA levels determined by RT-PCR, followed by quantitation of radioactivity by phosphor-stimulated luminescence and calibrated to the amount of β-actin mRNA (set as 1). Bars indicate means ± 1 SD.

The results of this experiment also have an important bearing on the possible mechanism of diabetogenicity of the NIH diet. If this diet provided only an antigenic trigger for a self-perpetuating islet inflammatory process, a dose response, as observed here, would not be expected. Further evidence for a continuous and additive effect of dietary exposure comes from the timing studies, which revealed several important findings. Exposure to a diabetogenic diet between weaning and puberty (23–50 days), followed by feeding a protective HC diet, resulted in a diabetes incidence less than half that of rats fed the NIH diet throughout the experiment, and the insulitis grade was significantly lower than in NIH-fed rats (Fig. 3, Table 1). Conversely, feeding a protective HC diet between weaning and puberty and then switching to the NIH diet produced the same diabetes incidence at the same rate as constant exposure to the NIH diet from weaning, except that the age at onset was delayed by 28 days (Fig. 3, Table 1). In animals fed the HC diet from weaning to 100 days and then switched to the diabetogenic NIH diet, diabetes frequency was lower than in NIH-fed rats, and the histological picture was similar to that in animals fed the HC diet. This is an important point showing that age 50–100 days, corresponding to the period of early puberty to late adolescence, is a critical time for dietary modification of diabetes. Bearing in mind that we find a dose response to food diabetogens, this result does not rule out a role for exposure at an earlier age if high concentrations of food diabetogens are encountered. This finding is in keeping with previous results showing that when HC or other low-diabetogen diets are fed from weaning, long past infancy in the rat, the protective effect is still apparent (32).

Taken together, these results do not support the notion of a single triggering dietary exposure in the infancy of BB rats as the primary diet-diabetes interaction. Rather, they point to a dose-dependent interaction affecting islet inflammation and diabetes that may still be modified as late as puberty in the rat. It is of interest that, in the NOD mouse, where diabetes development is much slower and occurs well after adolescence, delayed introduction of a diabetogenic, mainly wheat-based diet also delayed or prevented diabetes onset (37,41,43). Hence the dietary promotion of diabetes development is not restricted to infancy in two different animal species; this may also be the case in humans.

Compared with DR-BB rats, the growth of the pancreas in DP-BB rats fed cereal-based diets is retarded as early as age 30 days, as evidenced by a reduction of 50% in pancreatic insulin content; by 45 days, the weight of the pancreas was also reduced by 21% (44). This group subsequently reported that insulitis and diabetes in the DP-BB rat were preceded by decreased β-cell and pancreas volume, and they concluded that growth or replication of β-cells was affected before diabetes onset (45). The present findings—that, in comparison with young, 41-day-old NIH-fed DP-BB rats, age-matched DP-BB rats fed a protective HC diet had larger total islet area, similar to HC-fed or NIH-fed BBc rats—indicate there may be a fundamental diet-induced change in β-cell growth, turnover, or apoptosis before classic insulitis is apparent. Such an effect may also occur at the level of non-β-cells. Importantly, there was a diet-induced change in total islet area in DP-BB but not in BBc rats. This is the first indication that there are major, early, diet-induced modifications of islet cell mass in DP-BB rats. We did not investigate the effect of these diets on islet function per se. However, our results indicate that further studies are warranted to examine how food diabetogens might alter islet homeostasis and function. It is known from autopsy and clinical ultrasonography studies in children with IDDM that weight and size of the pancreas is decreased compared with that of healthy controls (46), but it is not known if islet mass or pancreas size is decreased in prediabetic humans.

Changes in islet cell activity may also alter the antigenic appearance of the β-cell (47). Furthermore, dietary treatment or fasting changes the immunogenicity of the islet in the rat as measured in the islet cell antibody assay (48), also suggesting that the activity or antigenicity of the target cell is an important feature of diabetes pathogenesis (49). Previous findings showed that hyperexpression of MHC class I molecules occurs before classic insulitis and is present even in silica-treated, macrophage-inhibited DP-BB rats fed a diabetogenic, cereal-based diet from weaning (22). Other treatments that are known to affect β-cell activity (such as daily insulin injections from 50 to 142 days [50] and glucose injection in the neonatal period [51]) are postulated to prevent diabetes by dampening or enhancing target cell antigenicity. Taken together, these data suggest that there may be important early diet-induced effects on the target cells that change their antigenicity even after silica treatment, a condition known to inhibit insulitis (52).

It has been suggested that diabetes is a Th1 cell–mediated disease (53). Considering the inhibitory effect of an HC diet
not only on diabetes but also on the frequency and severity of insulitis, it was important to determine whether there were diet-induced changes in pancreatic cytokines. The cytokine pattern from pancreases of NIH-fed DP-BB rats age 70 days showed high IFN-γ, characteristic of Th1 cells, whereas the reduced IFN-γ and raised levels of TGF-β indicated that Th2 cells predominated in the pancreas of HC-fed DP-BB rats (Fig. 4). Our findings in NIH-fed DP-BB rats are in keeping with reports describing a Th1 cytokine pattern in pancreas (53), pancreatic islets (54), and islet-infiltrating leukocytes (55) of BB rats fed either the NIH diet or other similar standard rodent diets. These analyses were based on cytokine mRNA measurements rather than on detection of cytokine protein. However, in inflamed islets of prediabetic NOD mice, a close correlation of cytokine gene expression and immunostaining was reported (56). That diabetes in the BB rat is mediated by Th1 cells is further supported by another report in which injection of monoclonal antibodies against IFN-γ prevented BB rat diabetes (57). In addition to an early effect on islet area, the feeding of an HC diet not only inhibited insulitis (12,13) but was associated with a Th2 cytokine pattern. Thus the major effects of the HC diet in preventing a large proportion of DP-BB rat diabetes are to 1) inhibit the usual food diabetogen-related increase in MHC class I antigen expression (22), 2) maintain islet cell area at a level similar to control rats, 3) decrease infiltration of leukocytes into the islets, and 4) redirect the remainder of the inflammatory process in HC-fed rats from a Th1 cell reaction toward a nondestructive, Th2 reaction. This interpretation is supported by the changes in cytokine patterns associated with the groups where diets were switched at day 50 (Fig. 5). Thus replacement of the diabetogenic NIH diet at 50 days with a low-diabetogen diet enhanced the levels of Th2-associated cytokines without affecting IFN-γ (Fig. 5).

It should be noted that these observations are based on studies of cytokine mRNA patterns in total pancreas RNA. We discontinued a parallel analysis of islet RNA because of the decreased yield of islets isolated from BB rats with severe insulitis, and because of the loss of periductular and peril-insular infiltrates during washing procedures. In the BB rat, inflammatory foci are also seen in exocrine tissue (57a). Hence the cytokine mRNA pattern in total pancreas reflects the inflammatory process at a more general level. Still, we observed a close correlation between cytokine mRNA pattern and the mean insulitis score in individual BB rats (128); \( r = 0.78, P < 0.001 \).

Interestingly, in another food-induced autoimmune condition, celiac disease, the majority of patients display an HLA-DQ2 association similar, but not identical, to that in IDDM. When T-cell clones from these patients' intestinal lesions were stimulated with wheat gluten peptides presented by B-cells, all the clones secreted IFN-γ with some tumor necrosis factor, indicating a Th1 type reaction in the mucosa (58). In another gut inflammatory condition associated with a Th1 cytokine pattern, Crohn's disease, elemental and protein hydrolysate diets have been used to induce lasting remission (59). In addition, the possibility that celiac disease may be the result of damage caused by wheat gluten reactive T-cells at the site of the lesion in the gut suggests intriguing similarities with respect to our finding that diabetes can be induced in the DP-BB rat by feeding a wheat gluten–based diet (15). All these data point to a pivotal role for diet and the gut immune system in modifying certain autoimmune diseases (60).

We conclude that diabetogenic agents in the diet are an essential part of the pathogenesis of IDDM in the DP-BB rat. Diet is also a major factor in determining diabetes outcome in NOD mice (37–43), and is probably an important determinant of diabetes outcome in humans as well. The present studies demonstrate that the dietary control of diabetogenesis is not restricted to early infancy in a trigger-like process, but is cumulative over time. Early in the pathogenic process, the dietary effect is at the level of islet cell homeostasis followed by direction of the autoimmune attack on the islets toward a destructive Th1 or a less damaging Th2 reaction at an age when the interaction between diet and diabetes susceptibility is the most crucial—that is, during puberty. The influence of food in enhancing or inhibiting the expression of diabetes is therefore a time- and dose-dependent interaction involving at least two essential elements: 1) changes in the target β-cells as reflected by altered islet area and antigenicity (22) followed by 2) a shift in the balance between Th1 and Th2 cells in the tissue undergoing autoimmune attack.

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APPENDIX: TERMINOLOGY

For terminology to describe the diabetes-inducing potential of foods and diets, we refer to diabetogenic, nondiabetogenic, or low-diabetogenic foods or food sources to differentiate the diabetes frequency we observe when these materials are fed to genetically susceptible DP-BB rats that develop spontaneous diabetes (13,14). For these foods to induce or modify diabetes outcome, they must be fed to diabetes-prone individuals and to our knowledge do not induce diabetes in the absence of a genetic predisposition to IDDM. A similar terminology is used in celiac disease, a known food-induced, autoimmune, gut enteropathy in which the so-called celiac toxic activity of wheat and certain other prolamins is required to observe the characteristic alterations in jejunal histology and accompanying symptoms that occur in genetically susceptible individuals, but not in the majority of the population.

REFERENCES

31. Scott FW: AAP recommendations on cow milk, soy and early infant feeding.


51. Rabinovich A, Suarez-Finanz W, El-Sheikh A, Sorenson O, Power RF: Cytokine gene expression in pancreatic islet-infiltrating leukocytes of BB rats: expression of Th1 cytokines correlates with b-cell destructive insulitis and IDDM. *Diabetes* 45:749-754, 1996.


58. Rabinovich A, Suarez-Finanz W, El-Sheikh A, Sorenson O, Power RF: Cytokine gene expression in pancreatic islet-infiltrating leukocytes of BB rats: expression of Th1 cytokines correlates with b-cell destructive insulitis and IDDM. *Diabetes* 45:749-754, 1996.