**Enteropathy precedes type 1 diabetes in the BB rat**

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Background and aims: There is increasing evidence implicating intestinal immune responses to dietary proteins in the pathogenesis of type 1 autoimmune diabetes (T1D). Here we investigated the association between intestinal pathology and dietary factors in T1D by examining the mucosal architecture in the BB rat model.

Methods: BB control (BBc) and diabetes prone (BBdp) rats were fed either a diabetes retardant hydrolysed casein based diet or one of two cereal based diets that promote the development of diabetes. Intestinal architecture was assessed in the jejunum by microdissection, histology, and immunohistology, and by measuring peroxidase activity and brush border invertase levels.

Results: Enteropathy was present in BBdp rats soon after weaning, as assessed by increases in crypt length and in the proliferative activity of crypt epithelial cells in the jejunum, and this remained constant until 120 days of age. There was also a decrease in invertase activity, as well as increased numbers of intraepithelial lymphocytes, increased levels of mucosal peroxidase activity, and infiltration of the mucosa by CD4+ T lymphocytes. Equivalent enteropathy was present at all times in BBdp rats and was not influenced by the nature of the diet or by thymectomy at three weeks at age, procedures which prevent the development of diabetes.

Conclusion: Enteropathy is a consistent feature in the diabetes prone BB rat but it precedes the onset of insulin and appears to be due to mechanisms distinct from those which cause diabetes. The beneficial effects of the diabetes retardant hydrolysed casein diet on diabetes are not due to an effect on intestinal architecture per se but mucosal damage may be necessary for the development of autoimmunity in the pancreas.

**MATERIALS AND METHODS**

**Animals**

Male and female diabetes prone BioBreeding (BBdp) and control BB rats (BBc) used in the dietary studies were obtained from the Animal Resources Division of Health Canada (Ottawa, Canada). For the thymectomy experiments, diabetes prone BB (BBDP/Gro) and diabetes resistant (BBDR/Gro) rats obtained originally from the University of Massachusetts (Worcester, Massachusetts, USA) were bred at the central animal facility of the University of Groningen. All animals were raised under specific pathogen free conditions and weaned at 23 days of age. Animals were given free access to food and water. Experiments were performed at 120 days of age, and animals were killed by cervical dislocation. All procedures were carried out in accordance with the guidelines of the Council of Animal Care of the Canadian Government. As a first step towards this aim, here we have examined for the presence of enteropathy in BB control (BBc) and diabetes prone (BBdp) rats and have determined the effects of diets with different diabetes inducing potential.

**Abbreviations:** T1D, type 1 autoimmune diabetes; BBc, BB control rats; BBdp, BB diabetes prone rats; ITG, tissue transglutaminate; WG, wheat gluten; HC, hydrolysed casein; CCP, crypt cell production rate; IEL, intraepithelial lymphocytes; DC, dendritic cell.
approved by the Animal Care Committees at each institution and the principles of laboratory animal care were observed, as described by the Canadian Council on Animal Care or the Dutch law on the use of experimental animals.

Diets

In the animal studies performed in Ottawa, rats were fed an NTP-2000 diet (NTP) (Zeigler Bros., Gardiners, Pennsylvania, USA) that is mainly plant-based (milk free) with wheat as the major component (37%), and containing approximately 14.6% protein, 8.2% fat, 9.9% crude fibre, 52% carbohydrate, and 10.7% moisture. The diet was irradiated before use and contained low levels of chemical and microbial contaminants. Two isocaloric and isonitrogenous semi purified test diets were used: a wheat gluten (WG) diet consisting of 22.5% wheat gluten (ICN Biochemicals, Cleveland, Ohio, USA) and a hydrolysed casein (HC) diet containing 20.0% 14.6% protein, 8.2% fat, 9.9% crude fibre, 52% carbohydrate, and 10.7% moisture. The diet was irradiated before use and contained low levels of chemical and microbial contaminants. Two isocaloric and isonitrogenous semi purified test diets were used: a wheat gluten (WG) diet consisting of 22.5% wheat gluten (ICN Biochemicals, Cleveland, Ohio, USA) and a hydrolysed casein (HC) diet containing 20.0% wheat gluten (Sigma) and 300 μM hydrogen peroxide (Merck, Darmstadt, Germany). Change in absorbance at 655 nm was measured and 1 unit of peroxidase activity was defined as 1 absorbance unit increase per minute under linear conditions.

Thymectomy

BBDp/Gro and BBDR/Gro rats were thymectomised at three weeks of age, as described previously. Completeness of thymectomy was verified by the absence of circulating thymic migrants in peripheral blood and by anatomical inspection at necropsy. Control rats were subjected to sham thymectomy at the same age.

Immunohistochemistry

Sections of small intestine (6–8 μm) were cut and mounted on to poly-L-lysine coated microscope slides which were air dried and stored at −20°C until required. Sections were brought to room temperature and fixed in 100% ethanol for 15 minutes. Endogenous peroxidase activity was quenched by three changes of 3% H2O2 and 0.1% Na2S in phosphate buffered saline for five minutes before blocking with an avidin-biotin blocking kit (Vector, Burlingame, California, USA). Sections were then incubated with purified anti-CD4 (OX-38), purified anti-CD8 (OX-8), purified anti-CD45R (HIS24), or α5 integrin (OX-62), all diluted 1:100, or with FITC conjugated anti-RU1B (OX-6) diluted 1:200 (all from Vector, USA). Sections were then incubated with purified anti-CD4 (OX-38), purified anti-CD8 (OX-8), purified anti-CD45R (HIS24), or α5 integrin (OX-62), all diluted 1:100, or with FITC conjugated anti-RU1B (OX-6) diluted 1:200 (all from PharMingen, Cowley, Oxford, UK) for 60 minutes. Antibody dilutions were made in a 0.1% bovine serum albumin BSA. T cell receptor β chain was detected using a supernatant containing the purified antibody which was a kind gift from Lesley Cousins (Sir William Dunn School of Pathology, Oxford, UK). Purified antibodies were amplified using the TSA Biotyn System (PerkinElmer Life Sciences, Wellesley, Massachusetts, USA) according to the manufacturer’s protocol and visualised using streptavidin Alexa Fluor 488 or 647 (Molecular Probes, Paisley, UK). Slides were mounted in Vectashield containing DAPI (Vector). Colour images of the tissue sections were captured using a Hamamatsu Orca ER Digital CCD camera attached to an Olympus BX50 microscope and were then processed using Openlab 3.1 software (Improvision, UK).

Statistical analysis

Comparisons between groups were performed using a one way ANOVA, Student’s t test, or the Wilcoxon rank sum test. CPR was compared using covariance analysis; p values less than 0.05 were considered significant.

RESULTS

Influence of diet on diabetes

The mean incidence of diabetes in BBdp rats in the Ottawa colony fed the standard NTP diet has remained constant over the past five years (mean 65.3 (SD 14.9%); total of 169 rats). Rats fed the WG diet have a similar incidence of diabetes (50.6 (11.1%); n = 282) whereas those fed the HC diet are protected from diabetes to a considerable extent (18.8 (10.6%); n = 322; p<0.001).

Development of enteropathy in diabetes prone rats

Mucosal architecture was measured in Bbc and BBdp rats from day 10 until 120 days of age. This spectrum covers the time before weaning, the period before classic insulitis, and
Intestinal pathology in BB rats

**NTP diet (5–8 rats/group).** NTP fed BBdp rats also had significant villus短ening at this time but this was not seen at other time points.

Significant enteropathy was present in the jejunum of BBdp rats fed the diabetes promoting WG and NTP diets at all times from 30 days of age onwards (figs 1, 3). The pathology consisted of marked crypt lengthening and increased numbers of mitotic bodies compared with the equivalent values in age matched BBc. All aspects of mucosal architecture were normal in BBdp rats examined before weaning at 10 days of age but after crypt hypertrophy and hyperplasia had appeared, these features remained constant until the experiment finished at 120 days. Increases in crypt length and mitotic activity were seen between days 10 and 30 in all rats, including BBc rats, features consistent with the physiological effects of weaning on intestinal maturation. A further early indicator of immunologically mediated enteropathy in the small intestine is an increased density of IELs. To examine if this also occurred in the BBdp rat, separate groups of BBdp and BBc rats were examined at the earliest time point when enteropathy was apparent (day 30). At this time, BBdp rats had a significant increase in the number of IEL in the jejunum compared with BBc controls (fig 4A).

Immunohistological analysis of jejunal sections taken from BBc rats on day 70 showed the expected pattern of lymphoid cells, with CD4+ cells in the lamina propria and CD8+ cells in both the lamina propria and epithelium (fig 5). Staining for the αβ T cell receptor confirmed that these CD4+ and CD8+ cells were T cells (data not shown). In addition, class II MHC+ cells were abundant in the lamina propria and double staining for the αβ integrin indicated that many of these were dendritic cells (DCs) (fig 5). In comparison, BBdp rats fed the NTP diet had markedly increased infiltration of

**Figure 1**  Diet and enteropathy in the BB rat. Mucosal architecture in the jejunum of BB diabetes prone (BBdp) and control (BBc) rats fed diabetes promoting (NTP, wheat gluten (WG)) or protective (hydrolysed casein (HC)) diets, assessed by crypt length (A) and number of mitotic figures in the crypts (B). Samples of jejunum were analysed by microdissection from 10–120 days of age and the results shown are means (SD) for BBc and BBdp rats, *p<0.05, **p<0.01, BBc versus BBdp; t*<0.05, BBdp rats fed HC diet versus BBc rats fed NTP diet; t**<0.05, BBdp fed HC diet versus BBdp fed NTP diet (5–8 rats/group).

**Figure 2**  Diet and enteropathy in the BB rat. Villus length in the jejunum of BB diabetes prone (BBdp) and control (BBc) rats fed diabetes promoting (NTP, wheat gluten (WG)) or protective (hydrolysed casein (HC)) diets. Samples of jejunum were analysed by microdissection from 10–120 days of age. The results shown are means (SD) for BBc and BBdp rats. *p<0.05, **p<0.01, BBc versus BBdp (5–8 rats per group).
lamina propria by CD4+ T cells but a virtual absence of CD8+ T cells and of class II MHC+ cells. αE integrin expressing cells were present in BBdp mucosa, but in reduced numbers compared with BBc, suggesting a deficiency of total DC numbers. There was a complete lack of class II MHC expression by these cells (fig 5). No class II MHC expression was found in the epithelium of any rat. The presence of intestinal inflammation in diabetes prone rats was underlined by an increase in the activity of the peroxidase enzyme associated with phagocytic cells. This was seen by day 30 (data not shown) and was even more marked by day 95 when BBdp rats had a sevenfold increase in levels of duodenal peroxidase compared with BBc controls (fig 4B; mean 362.3 (SEM 126.3) v 50.6 (13.8) mU/mg; p<0.02 Wilcoxon rank sum test). Together, these results indicate that there is an immunologically mediated enteropathy in diabetes prone BB rats.

Effects of diet on diabetes associated enteropathy
To explore whether these measures of enteropathy in BBdp rats were influenced by diets with different diabetes promoting potential, we compared indices of mucosal architecture in rats fed the protective HC diet with those fed the NTP or WG diets. Mucosal architecture in BBc rats was markedly influenced by the composition of the diets as throughout the study, animals fed the HC diet had significantly smaller crypts and generally lower CCPRs than BBc rats fed the NTP and WG diets (fig 1). In contrast, the nature of the weaning diet had little or no effect on the development of enteropathy in BBdp rats. As with rats fed the NTP or WG diets, significant increases in crypt length and mitotic activity were found in BBdp rats fed the protective HC diet compared with their equivalent controls at all times after day 30 (fig 1). Furthermore, there were no differences in villus length in BBdp rats fed a diabetes promoting or protective diet (fig 2), or in the immunohistological changes in CD4+ T cells, CD8+ T cells, or class II MHC+ cells seen in BBdp rats (fig 5).

Enteropathy in diabetic rats is associated with functional abnormalities in epithelial enzymes
To determine whether the enteropathy in BBdp rats also produced functional changes in the intestine and if these were influenced by diet, we measured levels of brush border invertase activity.

In BBc rats, duodenal invertase activity showed a sharp rise between day 10 and 30 and a late decrease between days 95 and 120, irrespective of diet (fig 6). At day 10, no significant difference in invertase activity was observed between BBc and BBdp rats but from day 30 onwards, invertase levels were consistently lower in BBdp rats fed the diabetes promoting NTP and WG diets or the protective HC diets than in equivalent BBc rats. These differences were statistically significant at days 30 and 70 in rats fed the NTP or HC diet and at day 70 in animals fed the WG diet (fig 6). No significant differences were found between invertase activity in BBc rats fed the HC diet and those fed either the NTP or WG diet at any time.

Effects of thymectomy on enteropathy and diabetes in BB rats
To examine the relationship between diabetes and enteropathy further, we used high incidence BBDP/Gro rats:
90–95% of these rats fed a standard cereal based diet develop T1D by the age of 10–14 weeks, with histological insulitis being observed from approximately eight weeks. The incidence of diabetes in these animals is reduced to 5% by thymectomy at three weeks of age and we therefore examined the effects of thymectomy on enteropathy. Mucosal architecture was measured in thymectomised and sham thymectomised BBDP/Gro or BBDR/Gro rats during the prediabetic phase (day 59) and around the time of onset of diabetes on day 84. Medium to severe insulitis was observed in BBDP/Gro examined at 84 days but no insulitis was found in any of the thymectomised BBDP/Gro rats examined at 59 and 84 days nor in any of the control BB rats (data not shown).

As in the Ottawa colony, sham thymectomised BBDP/Gro rats developed enteropathy during the prediabetic period, with significant crypt hypertrophy compared with BBDR/Gro controls on day 59 (fig 7). On day 84, there was also an increase in entering activity in the proximal gut of BBDP/NTP and BBDR/NTP rats compared with BBDP/HCP and BBDR/HCP rats (fig 6).

![Figure 5](image1.png)  
**Figure 5** Diet and enteropathy in the BB rat. Immunohistological appearance of the jejunum in 70 day old BB diabetes prone (BBdp) and control (BBc) rats fed hydrolysed casein (HC) and NTP diets. Frozen sections were stained for CD4, CD8, and class II MHC cells using Alex Fluor 488 (green) and for integrin using Alex Fluor 647 (red). Nuclei were stained with DAPI (blue) (x200 magnification).

![Figure 6](image2.png)  
**Figure 6** Diet and enteropathy in the BB rat. Invertase activity in the proximal gut of BB diabetes prone (BBdp) and control (BBc) rats fed NTP, wheat gluten (WG), or hydrolysed casein (HC) diets, determined as described in material and methods. The results shown are means (SEM) for 12 rats per group on day 10, and 3–5 per group for each remaining time point (*p<0.05, **p<0.01).
increase in CCPR, as measured by a metaphase arrest technique. A similar pattern of intestinal pathology was observed in thymectomised BBDR/Gro rats, with significant increases in crypt length on both days 59 and 84. Thymectomy had no effect on mucosal architecture in BBDR/Gro control rats. Thus thymectomy had no effect on enteropathy despite its ability to modify diabetes outcome in BBDR/Gro rats.

**DISCUSSION**

The results of this study show that enteropathy is a consistent feature of the BB rat model of T1D but indicate that this is not influenced by the complexity of the diet. Enteropathy in diabetes prone BB rats consisted of crypt hypertrophy and hyperplasia, as well as an increase in the proportion of IELs, a decrease in brush border enzyme activity, and increased hyperplasia, as well as an increase in the proportion of IELs, a decrease in brush border enzyme activity, and increased infiltration of the mucosa by CD4^+^ T cells. Furthermore, the activity of peroxidase was higher in the duodenal mucosa of BBdp rats compared with that of control rats. These features are characteristic of immunologically mediated pathologies in the small intestine, specifically those involving T cells, including coeliac disease,^34^ parasite infections,^35^ the early stages of Crohn’s disease and intestinal graft versus host disease.^36^-^38^ In these cases, tissue damage is believed to be caused mainly by cytokines such as interferon γ and/or tumour necrosis factor α released during a local CD4^+^ T cell mediated immune response. Although the features of the enteropathy we found in BBdp rats were similar to those seen in coeliac disease, villus atrophy was not a marked feature in BB rats and more dramatic increases in IEL numbers are usually seen in coeliac patients. This could suggest that the enteropathy in T1D is simply a less severe form of the same type of immunopathology. However, it should be noted that once present, the enteropathy did not seem to progress to more severe pathology in the BB rats, as might be expected if it simply represented an early stage in a continuum of disease. Therefore, it may reflect a distinct, if overlapping, set of mechanisms.

As the coeliac disease-like enteropathy that occurs in humans with T1D has only been reported in patients with established diabetes, the cause and effect relationship between the two conditions has been uncertain. Our results showed that crypt hypertrophy and hyperplasia appeared between 10 and 30 days of life in BBdp rats, then remained constant for the remainder of the study. As the animals did not develop clinical diabetes until after ~60 days of age, our findings indicate that the enteropathy is not merely a side effect of diabetes. Furthermore, enteropathy was seen in thymectomised BBdp rats that did not go on to develop insulitis or clinical diabetes, further eliminating the metabolic effects of diabetes as the causative agent of the gut abnormalities. In an attempt to explain these findings, we initially hypothesised that an aberrant T cell response to dietary protein antigen could be responsible for the enteropathy, which in turn would be a prerequisite for diabetes development. The beneficial effects of low antigen diets that we^1^ and others^1^ have found in T1D would also be consistent with this idea. Furthermore, using a wheat cDNA expression library, we have recently identified a wheat storage globulin, Gib1, that is recognised by antibodies from diabetic BBdp rats and which shares homology with tight junction proteins from the intestinal epithelium. A hydrolysed casein based diet inhibited the appearance of clinical diabetes compared with rats fed more complex, mainly wheat based, diets. In addition, the mucosal architecture in control rats fed hydrolysed casein was less mature than in rats fed the other diets. However, the protective diet had no effect on enteropathy in BBdp rats, indicating that the effects of diet on diabetes are not related to an ability to prevent enteropathy per se.

The antigenic stimulus for intestinal pathology remains unknown. One possibility could be that a local immune response against commensal bacteria leads to intestinal pathology, as occurs in experimental models of inflammatory bowel disease. However, enteropathy in BBdp rats and humans with T1D is mainly in the upper small intestine where bacteria are rare. Conversely, tissue damage in models of inflammatory bowel disease that require commensal bacteria is restricted to the colon. An alternative explanation is that there is an immune response against a self antigen that is shared by both the gut and pancreas. However, thymectomised BBDP rats developed enteropathy in the absence of insulitis, indicating that the intestinal pathology does not require an autoimmune response against the pancreatic islet. In addition, although the pancreas is derived from the embryonic gut during development, no specific cross reacting tissue antigen of this kind has been identified. Some diabetic patients have antibodies against tissue transglutaminase, an autoantigen which is implicated in coeliac disease and which could also be expressed in the pancreas. However, production of these antibodies is entirely dependent on the presence of gluten in the diet and therefore this type of response should be related to the composition of the diet, which was not the case for enteropathy in BBdp rats.
Our experiments on thymectomised BB rats demonstrate that different effector mechanisms are responsible for the enteropathy and diabetes. T1D is now considered to occur because a defect in regulatory lymphocyte activity permits autoreactive CD4+ and CD8+ T cells to infiltrate and damage the islet. Thymectomy at three weeks of age prevents diabetes from developing in BB rats due to removal of effector CD8+ T cells but enteropathy was still present in these animals. In addition, immunohistological analysis revealed that CD8+ T cells and class II MHC+ cells were absent from the jejunum of the BBdp rat but there was a marked increase in CD4+ T cells in BBdp lamina propria. These findings are consistent with the known effects of lymphaopnea on CD8+ T cells and IEL in the BBdp rat and, together, support the idea that CD4+ T cells may be responsible for the associated enteropathy. A striking finding from the immunohistological analysis was the decreased numbers of class II MHC+ DC in BBdp rat mucosa, perhaps indicating abnormal maturation or activation of intestinal DC in these animals. There have been reports of abnormal maturation and functions of DC in both BBdp rats and NOD mice (67) (our unpublished observations). Thus it would be interesting to confirm our findings and to determine if DC dysfunction plays a role in the enteropathy or diabetes in the BB rat.

The fact that enteropathy is independent of diabetes raises the question of why enteropathy did not occur in Bbcr rats that share a large proportion of their genes. One of the best understood genetic differences between the two strains is the presence of the lymphopaenia (lyp) gene in diabetes prone BB rats and it is possible that diabetes and enteropathy in diabetes prone BB rats are independent consequences of the lyp mutation. The genetic association between lymphopaenia, diabetes, and enteropathy needs to be addressed in appropriate congenic BB rat lines. Depending on the results of this analysis, it may be that genetic susceptibility to enteropathy is one of the multiple factors that contributes to the pathogenesis of T1D in humans and it will be interesting to determine whether this feature is present in all experimental models of T1D.

In conclusion, this study has demonstrated that diabetes prone BB rats have a coeliac disease-like enteropathy that is established from an early age, providing further support for a link between the gut and the pathogenesis of T1D. The fact that diet and thymectomy, two external factors that have been shown to alter the course of diabetes, had no effect on gross measures of enteropathy suggests that these interventions do not prevent diabetes by restoring or preventing gut damage. This does not rule out the possibility that enteropathy is a constitutive trait that facilitates the development of diet induced diabetes. Future analysis of the pathogenesis of enteropathy may provide a useful clue to the genetic factors predisposing to T1D.

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REFERENCES

EDITOR’S QUIZ: GI SNAPSHOT

A waxing and waning skin rash

Clinical presentation

A 72 year old Caucasian female attended the Department of Dermatology with an 11 year history of elevated blood sugar (145 mg/dl) (fig 1). A computerised tomogram was performed (see fig 2). The patient was scheduled for surgery, and a distal pancreatectomy and splenectomy were performed. Examination of the rest of the abdomen was normal. The patient is free of disease after 24 month of follow up and dermal changes cleared seven days after surgery.

Question

What does computerised tomography show and how does it explain the longstanding rash?

Figure 1

Figure 2

Computerised tomography of the abdomen.

See page 1451 for answer

This case is submitted by:

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