

Antibiotic treatment partially protects against type 1 diabetes in the Bio-Breeding diabetes-prone rat. Is the gut flora involved in the development of type 1 diabetes?

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Abstract

Aims/hypothesis Accumulating data suggest that the gut immune system plays a role in the development of type 1 diabetes. The intestinal flora is essential for the development of the (gut) immune system and the establishment of tolerance. It has been reported that oral administration of food and bacterial antigens early in life suppresses later development of diabetes in the Bio-Breeding diabetes-prone (BB-DP) rat. This study was designed to investigate the possible relationship between the development of diabetes and the composition of intestinal flora.

Materials and methods The intestinal flora of BB-DP rats, a rat model for type 1 diabetes, was characterised long before the clinical onset of diabetes by fluorescent in situ hybridisation. In a separate experiment, BB-DP rats were treated with antibiotics and the effect on diabetes incidence and level of insulinitis was analysed.

Results We observed a difference in bacterial composition between rats that eventually did and those that did not develop diabetes. This difference was detectable long before clinical onset of the disease. Rats that did not develop diabetes at a later age displayed a lower amount of *Bacteroides* sp. Modulation of the intestinal flora through

antibiotic treatment decreased the incidence and delayed the onset of diabetes. A combination of antibiotic treatment and a protective hydrolysed casein diet completely prevented diabetes in the BB-DP rat.

Conclusions/interpretation Our data suggest that the intestinal flora is involved in the development of type 1 diabetes. Factors influencing composition of the intestinal flora could be a target for therapeutic intervention.

Keywords Antibiotics · BB-DP rats · Insulinitis · Intestinal flora · Type 1 diabetes

Abbreviations

BB-DP Bio-Breeding diabetes-prone
CON Conventional plant-based
FISH Fluorescent in situ hybridisation
HC Hydrolysed casein

Introduction

Type 1 diabetes results from complex interactions between genetic and environmentally determined factors. Experiments performed in animal models for type 1 diabetes have shown that diet can influence the diabetes outcome. Plant-based diets appear to be the most ‘diabetogenic’ and this seems to be dependent on dose (‘antigenic load’). Hydrolysed casein (HC) diet, a diet in which the sole protein source is hydrolysed casein, has a protective effect on diabetes development in Bio-Breeding diabetes-prone (BB-DP) rats [1]. Several mechanisms for the protective effects of the HC diet have been described, such as Th2 skewing, islet neogenesis and inhibition of hyperexpression of MHC class I on beta cells [2]. In addition, a diabetes-preventive

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gluten-free diet has been shown to decrease the number of caecal bacteria in non-obese diabetic mice, suggesting that such diets may also exert their protective effect by changing the intestinal flora [3, 4].

The fact that some BB-DP rats do not develop diabetes suggests that other factors, besides genes and diet, play a role in the development of diabetes. The increasing prevalence of food allergies in Western societies has led to the hypothesis that increased hygienic conditions throughout life can disturb normal tolerance induction to otherwise harmless food proteins and other (inhaled) antigens. Previous studies have shown that modulation of antigen exposure early in life can change the diabetes incidence in BB-DP rats [5, 6]. Furthermore, the anti-staphylococcal drug fusidin was able to decrease the diabetes incidence in BB-DP rats [7].

In order to determine if the intestinal flora plays a role in the development of type 1 diabetes in the BB-DP rat, we analysed the intestinal flora of BB-DP rats that became diabetic and of BB-DP rats that did not become diabetic. This analysis was done long before the onset of diabetes in affected animals. In a separate experiment, we also modulated the intestinal flora by antibiotic treatment and determined the effect on the diabetes incidence and level of insulinitis.

Materials and methods

Animals

BB-DP rats were derived from the Worcester BB-DP strain, but were bred in the Groningen animal facility (BBDPWor/Gro). In our colony, 80 to 90% of the BB-DP rats spontaneously develop diabetes from 65 days of age on a conventional plant-based (CON) diet. No sex-related differences are observed. An animal is considered diabetic when blood glucose levels exceed 15 mmol/l. Animals were treated in compliance with Dutch law on experimental animal care.

Experiment 1. Faecal flora composition of BB-DP rats before onset of diabetes

BB-DP rats, males and females, were group-housed (two or three/cage) under specific-pathogen-free conditions. Rats had free access to a CON diet (Rmh-B2181; Hope Farms, Woerden, the Netherlands) and water.

Fluorescent *in situ* hybridisation (FISH)

Faecal samples of 40-day-old BB-DP rats were dissolved in PBS, centrifuged at low speed and supernatants were fixed in 4% paraformaldehyde overnight at 4°C. Samples were washed and fixed in 96% ethanol for 2 h at 4°C.

Subsequently, samples were hybridised at 50°C with fluorescent (FITC)-labelled 16S rRNA-targeted oligonucleotide probes for different groups of bacteria (Eurogentec, Maastricht, the Netherlands) in hybridisation buffer (20 mmol/l Tris-HCl, 0.9 mol/l NaCl, 0.1% SDS, pH 7.2) overnight. The probes used were: Nonbact338 (negative control), Bact338 (universal), Bac303 (*Bacteroides* sp.), Erec482 (*Clostridium* sp.), Mib661 (mouse intestinal bacteria) and Lab158 (*Lactobacillus* sp.) [8, 9]. Prior to analysis, samples were washed with hybridisation buffer without SDS for 30 min at 50°C. Next, samples were washed with PBS, stained with propidium iodide (DNA stain) and analysed by flow cytometry [8].

Experiment 2. Antibiotic treatment of BB-DP rats

BB-DP rats, males and females, were group-housed (two or three/cage) under specific-pathogen-free conditions. Rats had free access to food and water (with or without antibiotics). BB-DP rats that received antibiotic treatment were kept in a laminar flow in filter-top cages.

Treatment groups

From weaning (day 21), two groups of BB-DP rats continuously received antibiotics in the drinking water (Bactrimel [sulphamethoxazole (1.2 g/l) and trimethoprim (240 mg/l)]; Roche, Woerden, the Netherlands) and colistine sulphate (1.0 g/l), decreasing various Gram-negative and Gram-positive species. One group of rats on antibiotic treatment received a CON diet ($n=8$), the other group received an HC diet ($n=10$) (a modification of the AIN-93G diet containing 20% hydrolysed casein as the source of amino acids [Solka-Floc; Teklad, Madison, WI, USA]). Two diet control groups that did not receive antibiotics were added (CON $n=14$; HC $n=15$).

FISH and fluorescent microscopy

In order to evaluate the effect of the antibiotic treatment on the amount of different bacteria, FISH was performed with the same set of probes as in Experiment 1. The bacteria hybridising with the probes were quantified by fluorescent microscopy.

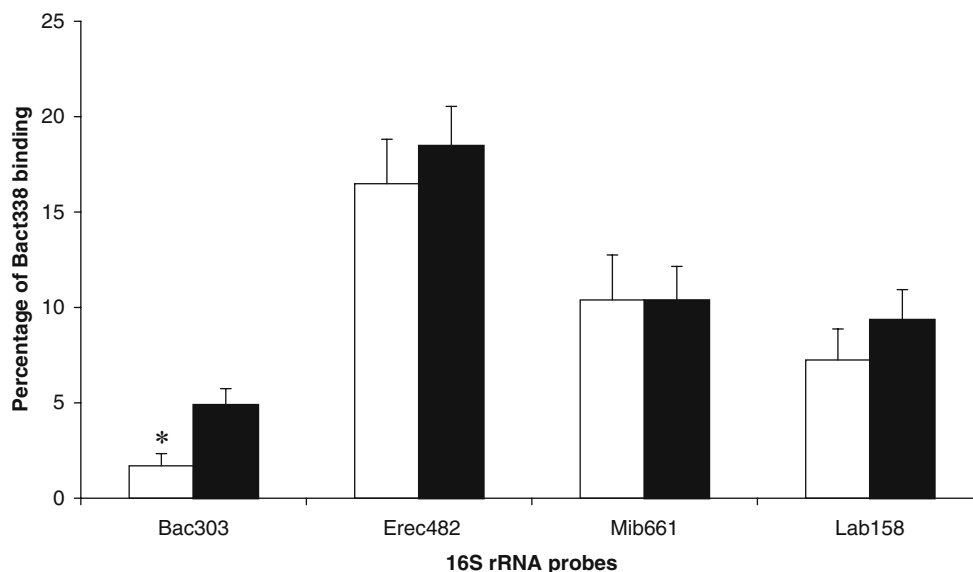
Insulinitis

Haematoxylin/eosin-stained paraffin sections of the pancreas were evaluated for insulinitis as previously described [4].

Statistics

Student's *t*-test was used for statistical analysis of the intestinal flora composition and statistical analysis of the

Fig. 1 Composition of the faecal flora of BB-DP rats (40 days old) expressed as a percentage of Bact338 binding. *Black bars* BB-DP rats that eventually developed diabetes ($n=22$). *White bars* BB-DP rats that did not develop diabetes ($n=13$). $*p=0.01$, Student's t -test, two-tailed. *Error bars*, SEM



day of diabetes onset. The Kaplan–Meier test for survival, log-rank, was used for analysis of the survival data.

Results

Experiment 1. Faecal flora composition of BB-DP rats before onset of diabetes

In a retrospective analysis, we investigated whether differences in faecal flora composition already existed, prior to the disease onset, between BB-DP rats that eventually developed diabetes and rats that did not develop diabetes. Figure 1 shows binding of the bacterial group-specific probes in faecal samples at the pre-diabetic timepoint (day

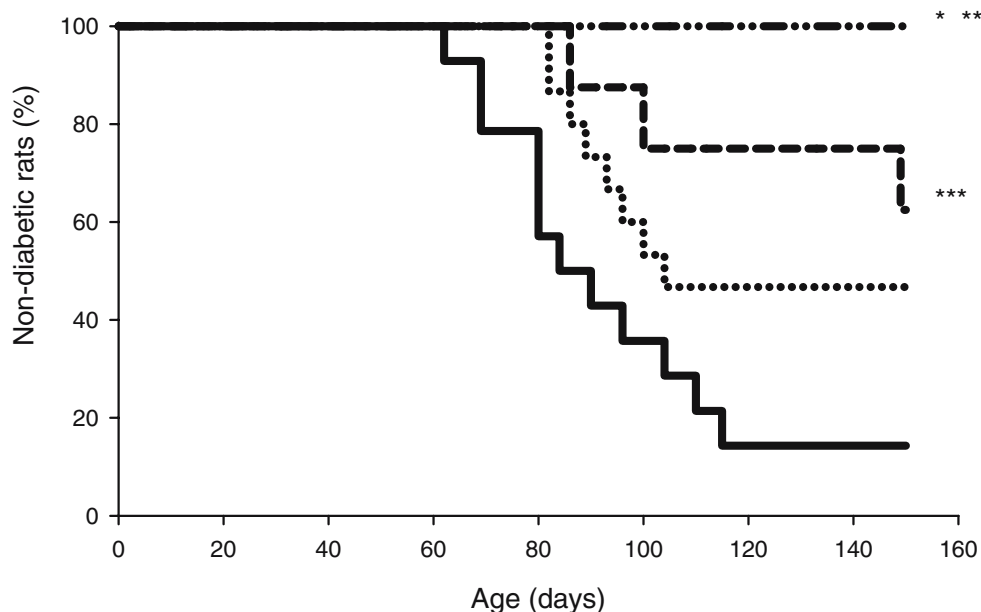
40 of age). The faecal samples, taken before clinical onset, of rats that later developed diabetes show a higher binding to the *Bacteroides* sp. group-specific probe than the rats that did not develop diabetes ($p=0.01$).

Experiment 2. Antibiotic treatment of BB-DP rats

Effectiveness of the antibiotic treatment

Antibiotic treatment lowered the amount of intestinal bacteria from 10^{10} /g faeces to 10^8 /g faeces as measured by counting Bact338 (universal 16S rRNA probe) positive events by fluorescent microscopy. Antibiotic treatment reduced binding to the specific probes. A reduction of 43,

Fig. 2 Diabetes incidence of different treatment groups. *Solid line* BB-DP rats receiving CON ($n=14$); *dotted line* BB-DP rats receiving HC diet ($n=15$); *dashed line* BB-DP rats on antibiotic treatment from weaning and receiving CON ($n=8$); *dot-dotted and dashed line* BB-DP rats on antibiotic treatment from weaning and receiving HC diet ($n=10$). $*p=0.01$ for the HC diet +antibiotic treatment compared with the HC diet alone; $**p=0.002$ for the HC diet +antibiotic treatment compared with CON alone; $***p=0.02$ for the CON+antibiotic treatment compared with CON alone (all by Kaplan–Meier test for survival, log-rank)



95, 92 and 93% was seen for Bac303, Erec482, Mib661 and Lab158, respectively (data not shown).

Antibiotic treatment and diabetes development

Figure 2 shows that control rats receiving only CON developed diabetes from 60 days of age and eventually 86% of the rats developed diabetes (12 out of 14 rats). However, if rats received antibiotics with the CON diet the diabetes incidence decreased to 37.5% (three out of eight rats) ($p=0.02$). Interestingly, antibiotic treatment also delayed diabetes onset by 30 days ($p=0.004$). In the HC diet group, 53% of the animals developed diabetes (8 out of 15 rats) ($p=0.08$). However, diabetes onset was significantly delayed by 25 days ($p=0.04$). Strikingly, combining antibiotic treatment and HC diet totally protected against diabetes in the BB-DP rat; none of the ten rats developed diabetes ($p=0.01$ compared with the diet control group; $p=0.002$ compared with the group receiving CON alone).

Insulinitis

All diabetic animals displayed severe insulinitis (data not shown), consistent with the finding that clinical diabetes becomes manifest when 80% of the islets are destroyed. The non-diabetic rats in all groups did not display insulinitis (score 1) or only very mild insulinitis (score 2) (data not shown).

Discussion

Our data strongly support the notion that the composition of the gut flora is indeed involved in the development of type 1 diabetes. The finding that the faecal flora differs before the onset of the disease between rats that will and those that will not develop diabetes suggests that the flora might have a causative rather than a secondary role in the pathology of diabetes.

Antibiotic modulation of the intestinal flora in our BB-DP rat model for type 1 diabetes delayed and lowered the diabetes incidence significantly. A combination of antibiotic treatment and HC diet was able to totally prevent diabetes in the BB-DP rat. Modulation of the intestinal flora by antibiotic treatment could lower the antigenic load and therefore reduce diabetes incidence. However, antibiotic treatment (fusidic acid or cyclosporin A) has also been shown to prevent the lymphocyte-stimulatory activities of the cytokines IL-1 and IL-6 [10]. Antibiotic treatment therefore could also have a direct effect on the pancreas. Furthermore, protective diets have been shown to alter the bacterial composition [3]. Similarly, we find differences in the intestinal flora before the onset of diabetes in

unmanipulated BB-DP rats between rats that will or will not develop diabetes eventually.

Modulating the composition of the intestinal flora by antibiotic treatment and/or HC diet selectively removes some commensals while others are able to grow, thereby not only reducing the overall antigenic load but reducing the antigenic load of a given specificity as well.

We propose that the (specific) antigenic load in the intestine is important for the outcome of diabetes and that this antigenic load has a different effect on the immune system in different periods in life. Analysis of the complex interactions between antigenic load, the composition of the intestinal flora and the (gut) immune system in type 1 diabetes could well lead to new insights into this disease. Furthermore, this knowledge could possibly provide an important basis for the development of future preventive measures.

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