DIABETES/METABOLISM RESEARCH AND REVIEWS
Diabetes Metab Res Rev 2008; **24**: 59–63.
Published online 2 July 2007 in Wiley InterScience (www.interscience.wiley.com) **DOI:** 10.1002/dmrr.748

RESEARCH ARTICLE

Gluten-free but also gluten-enriched (gluten+) diet prevent diabetes in NOD mice; the gluten enigma in type 1 diabetes

David P. Funda[1,2]*
Anne Kaas[2]
Helena Tlaskalová-Hogenová[1]
Karsten Buschard[2]

[1]*Division of Immunology and Gnotobiology, Institute of Microbiology, Czech Academy of Sciences, Prague, Czech Republic*

[2]*Bartholin Instituttet, Rigshospitalet, Copenhagen, Denmark*

*Correspondence to:
David P. Funda, Division of Immunology and Gnotobiology, Institute of Microbiology, Czech Academy of Sciences, Vídeňská 1083, 142 20, Prague 4, Czech Republic.
E-mail: funda@biomed.cas.cz

Received: 4 September 2006
Revised: 14 January 2007
Accepted: 18 March 2007

Abstract

Background Environmental factors such as nutrition or exposure to infections play a substantial role in the pathogenesis of type 1 diabetes (T1D). We have previously shown that gluten-free, non-purified diet largely prevented diabetes in non-obese diabetic (NOD) mice. In this study we tested hypothesis that early introduction of gluten-enriched (gluten+) diet may increase diabetes incidence in NOD mice.

Methods Standard, gluten-free, gluten+ modified Altromin diets and hydrolysed-casein-based Pregestimil diet were fed to NOD females and diabetes incidence was followed for 310 days. Insulitis score and numbers of gut mucosal lymphocytes were determined in non-diabetic animals.

Results A significantly lower diabetes incidence ($p < 0.0001$) was observed in NOD mice fed gluten-free diet (5.9%, $n = 34$) and Pregestimil diet (10%, $n = 30$) compared to mice on the standard Altromin diet (60.6%, $n = 33$). Surprisingly, gluten+ diet also prevented diabetes incidence, even at the level found with the gluten-free diet ($p < 0.0001$, 5.9%, $n = 34$). The minority of mice, which developed diabetes on all the three diabetes-protective (gluten+, gluten-free, Pregestimil) diets, did that slightly later compared to those on the standard diet. Lower insulitis score compared to control mice was found in non-diabetic NOD mice on the gluten-free, and to a lesser extent also gluten+ and Pregestimil diets. No substantial differences in the number of $CD3^+$, TCR-$\gamma\delta^+$, and IgA^+ cells in the small intestine were documented.

Conclusions Gluten+ diet prevents diabetes in NOD mice at the level found with the non-purified gluten-free diet. Possible mechanisms of the enigmatic, dual effect of dietary gluten on the development of T1D are discussed.

Keywords gluten; gluten-free; type 1 diabetes; non-obese diabetic (NOD) mice; diet; environmental factors

Introduction

Environmental factors play a critical role in the development of T1D on a genetically susceptible background however, little is known about their exact identities as well as mechanism of action. High diabetes penetrance in non-obese diabetic (NOD) mice depends on the quality of the specific pathogen free (SPF) animal facilities [1]. Several studies in both BB rats and NOD mice documented that dietary components influence development of T1D [2–5].

In experimental animal models of T1D, highest diabetes incidence is achieved with non-purified, open formula diets (such as NIH-07, OG96), in which wheat flour is one of the major protein nutrient sources. It has been shown that dietary components allowing high penetrance of the disease are not carbohydrates but come from the protein plant fraction of non-purified diets [2,4,5]. While meat protein, corn starch, corn oil and with some controversy also milk proteins did not influence diabetes incidence, wheat gluten and soybean proteins were identified as major diabetogenic or rather diabetes-permissive dietary components [2–5].

Purified diets based on casein, hydrolysed casein (Pregestimil), hydrolysed lactalbumin or amino acids prevented T1D in NOD mice and BB rats [2,4,5]. Adding different potentially diabetogenic components to purified diet-base led often to insignificant or only partial restoration of the diabetogenic effect [4,5]. These results may be explained in same cases by late introduction of the diet and/or relatively low amount of the tested components (such as gluten) compared to its proportion in diabetogenic, non-purified formula. Recently, a well designed study in NOD mice documented that wheat and barley protein-free, non-purified Altromin diet supplemented with 4 g/100 g of gliadin partially restored diabetes incidence, but not to the level found with the diabetes-permissive standard Altromin diet [6].

Identification of putative diabetogenic or diabetes-permissive components is rather difficult due to the complexity of non-purified diets. That is why, we have previously tested a non-purified, gluten-free diet, which was prepared as a modification of the diabetogenic, non-purified standard Altromin diet, and in which all grain protein was replaced with meat protein while keeping the same amount of soybean and milk proteins as well as similar caloric value. This non-purified, gluten-free Altromin diet highly prevented diabetes incidence in NOD mice [7]. Although many studies documented dietary prevention of T1D in spontaneous animal models, no dietary manipulation has so far been able to increase diabetes incidence above the long-term app. 60–80% incidence found with standard, wheat-based, non-purified diets and within the same SPF animal facility.

On this background we asked whether by increasing the gluten content on account of previously reported non-diabetogenic meat protein and while keeping all other open formula components of a diet, one could yet increase the diabetes-permissive effect of the non-purified, standard Altromin diet. Thus, we investigated whether early introduction of gluten-enriched (gluten+) Altromin diet influences diabetes incidence, insulitis as well as the number of gut mucosal lymphocytes in NOD mice. Gluten-free, non-purified Altromin diet and hydrolysed-casein based Pregestimil hypoallergic infant formula were used as low diabetogenic (negative control) diets.

Materials and methods

Animals

Breeding pairs of inbred NOD mice were obtained from Taconic Europe A/S, Ry, Denmark, kept under SPF conditions (according to the FELASA guidelines) and had free access to acidified drinking water. The breeding pairs of NOD mice were fed one of the four different diets used in this study, so the experimental NOD female mice within each group were never exposed to other dietary regimen. First generation female offspring was used in this study and all four dietary groups of NOD females were observed for the diabetes incidence simultaneously. In all of these animal experiments, principles of animal care (NIH publication no. 85–23, revised 1985) and the national laws on Protection of Animals were followed.

Diets

The composition of the standard, non-purified 1434 Altromin diet as well as experimental diets is given in Table 1. Gluten-free and gluten+ modified Altromin diets were prepared as modifications of the standard non-purified 1434 Altromin diet (Altromin, Lage, Germany). Hydrolysed-casein based hypoallergic infant formula – Pregestimil (Mead Johnson, Bristol Myers Squibb Company, New York, NY) was used as another gluten-free (negative control) diet. The total protein content of the standard, gluten-free and gluten+ Altromin diets was 22.7, 22.9 and 22.8%, respectively. Pregestimil contains 14% of a protein equivalent in the form of extensively hydrolysed casein supplemented with L-cystine, L-tyrosine and L-tryptophan. The strategy for preparing the non-purified gluten-free Altromin diet was to keep the same content of milk and soybean proteins, which were previously reported as diabetogenic, and to replace the gluten-containing ingredients with meat protein whilst keeping a similar content of protein, fat, fiber and minerals. Similarly, the gluten+ Altromin diet was prepared by replacing meat and corn-starch protein with gluten (Sigma, St Louis, MO), while keeping the same content of cereal, milk and soybean proteins as in the standard Altromin diet. All three Altromin diets were equally supplemented with vitamins and minerals.

The gliadin content in all experimental diets was checked by a sensitive anti-gliadin ELISA kit (Immunotech, Beckman Coulter, Inc., CA) developed for testing gluten-free food products. While high gliadin content was detected in the standard Altromin (10 g/kg) and gluten+ modified diets (44 g/kg), the non-purified, gluten-free Altromin diet displayed gliadin content of 75 mg/kg; i.e. well bellow the cut of value of 200 mg/kg proposed for gluten-free food products for celiac disease patients [8,9]. No detectable gliadin values were found in the hydrolysed-casein based Pregestimil diet as the measured extinctions were bellow the sensitivity of the test.

DOI: 10.1002/dmrr

Table 1. Composition of test diets (g/100 g)

Ingredients (g/100 g)	Standard Altromin 1434	Gluten-free Altromin	Gluten+ Altromin	Pregestimil
Total Protein	22.7	22.9	22.8	14 (equivalent)
Meat protein	8.4	15.3	–	–
Cereal protein	3.5	–	3.5	–
Gluten (Sigma)	–	–	11.8	–
Corn starch	3.2	–	–	–
Soybean protein	6.5	6.5	6.5	–
Milk protein	1	1	1	–
Crude Fat	8.2	8.2	8.2	28
Fibre	3.1	3	3	–
Minerals	5.5	5.4	5.4	2.9
Lysin	1.3	1.3	0.8	1.1
Methionin	0.6	0.6	0.4	0.4
Threonin	0.8	0.8	0.7	0.6
Tryptophan	0.2	0.2	0.2	0.2
Glutamic acid	2.8	1.5	6.2	3
Water	9.5	4.8	5.9	2
Energy (kcal/kg)	3 308	3644	3 503	5 000

The data given here are representative of three independent ELISA experiments (including ethanol extraction of gliadin from the diets). The assessment of the gluten+ diet was less accurate because the ELISA kit is primarily designed for detection of low gliadin concentrations.

Evaluation of diabetes incidence

Experimental NOD females were inspected daily for diabetes and from 80 days of age screened weekly for glycosuria with TestTape (Eli Lilly, IN). Glycaemia was determined with Glucometer Elite 3922 (Bayer AG, Zurich, Switzerland). Diagnosis of diabetes was based on a positive glycosuria test followed by blood glucose readings >12 mmol/L for three consecutive days. The date of the first positive glycaemia reading was used as the onset of diabetes. Mice were killed after the third positive glycaemia reading or at age of 310 days. None of the experimental animals died from other causes.

Histology and immunohistology

Haematoxylin and eosin-stained pancreas sections from non-diabetic animals were evaluated for the insulitis score using following scale: (1) intact islet, (2) peri-insulitis, (3) moderate insulitis (50% of the islet infiltrated), (4) severe insulitis (>50% of the islet infiltrated). At least 15 islets for each non-diabetic mouse were scored blind.

Immunohistochemical staining was performed on 7 μm frozen sections from proximal and distal jejunum as well as proximal ileum as described in [6]. At least five non-diabetic NOD mice from each dietary group were used. Following primary biotin-conjugated anti-TCR gamma/delta, clone GL3 (BD Pharmingen, San Jose, CA), anti-CD3, clone KT3 (Serotec, Oxford, UK), biotin-conjugated sheep anti-mouse IgA, IgM (The Binding Site Ltd., Birmingham, UK) and secondary biotin-conjugated $F(ab')_2$ fragment goat anti-rat (Jackson Immunoresearch Lab., West Grove, PA) antibodies were employed in this study. Positive cells were visualized by Cy3-conjugated streptavidin (Jackson Immunoresearch Lab.) under a fluorescence microscope (Olympus). Number of positive cells was blindly counted on two sections from each animal using a calibrated eyepiece and constant lengths of intestinal epithelium.

Statistical analysis

The cumulative diabetes incidence was assessed using the Kaplan–Meier estimation and log-rank test was used for comparisons between groups. Other results are expressed as mean ± SEM, and the level of significance ($p < 0.05$) was assayed by two-sample analysis (unpaired t-test).

Results

The four tested diets, including the powdered Pregestimil diet, produced similar weight gain as well as terminal weights. The cumulative diabetes incidence in SPF NOD female mice fed the non-purified standard diet, gluten-free and gluten+ modified Altromin diets as well as the hydrolysed casein-based Pregestimil diet is given in Figure 1.

While NOD females on the non-purified standard Altromin 1434 diet developed 60.6% ($n = 33$) diabetes incidence, both gluten-free modified Altromin diet and Pregestimil diet led to a statistically highly significant disease protection with diabetes frequency of 5.9% ($n = 34$, $p < 0.0001$) and 10% ($n = 30$, $p < 0.0001$), respectively. Surprisingly, the gluten+ modified Altromin diet also prevented development of diabetes in NOD

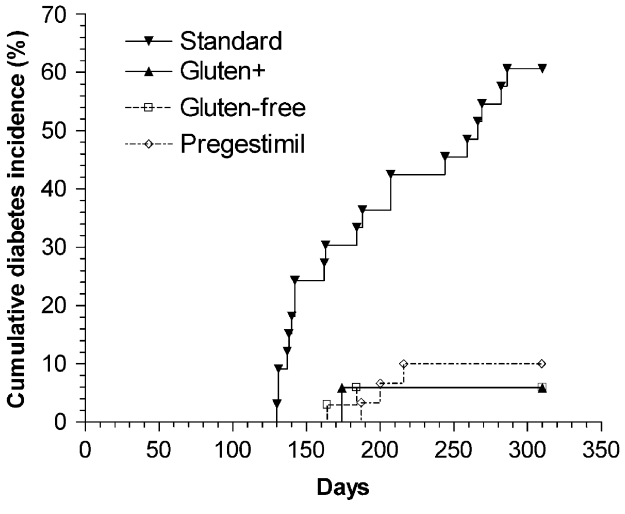

Figure 1. Cumulative diabetes incidence by 310 days of age in NOD female mice fed Standard Altromin 1434 ($n = 33$), gluten + ($n = 34$), gluten-free ($n = 34$) and Pregestimil ($n = 30$) diets. Breeding pairs of NOD mice were already fed the four tested diets, thus experimental animals were never exposed to other dietary components

DOI: 10.1002/dmrr

mice. Moreover it was as effective as the gluten-free Altromin diet (Figure 1.), producing only 5.9% ($n = 34$) diabetes incidence compared to 60.6% diabetes incidence on the standard diet ($n = 33$, $p < 0.0001$). Although not significant, later onset of diabetes was observed in NOD mice fed diabetes-protective gluten-free, gluten+ and Pregestimil diets compared to mice fed the standard diet (Figure 1.). No statistically significant differences in diabetes incidence were found among the three disease-protective diets.

Haematoxylin-eosin stained sections of pancreata were evaluated for islet lesions according to the insulitis score. Whilst terminal stage of insulitis was detected in diabetic animals, irrespective of the diet, many intact islets were found (scored) in non-diabetic mice at 310 days of age. The insulitis score was 1.8 ± 0.1; 2 ± 0.1; 2.1 ± 0.5 and 2.2 ± 0.1 for non-diabetic NOD mice on the gluten-free, gluten+, Pregestimil and standard diets, respectively. Lower insulitis score was found with all three disease-protective diets, but only the difference between NOD mice fed gluten-free *versus* standard diet was statistically significant at $p = 0.03$.

The effect of experimental diets on the gut mucosal immune system of NOD mice was assessed by enumeration of $CD3^+$, TCR-$\gamma\delta^+$ as well as IgA- and IgM-secreting cells in the proximal and distal jejunum as well as proximal ileum of non-diabetic animals. Only very few, scattered IgM-secreting cells were found within the lamina propria of all NOD mice. Within each dietary group, there were no substantial differences in the number of CD3-, TCR-$\gamma\delta$- as well as IgA- and IgM-positive cells among the proximal jejunum, distal jejunum and ileum. Similar numbers of $CD3^+$ (103 ± 10; 99 ± 9; 115 ± 9 *vs* 118 ± 12), TCR-$\gamma\delta^+$ (37 ± 2; 37 ± 4; 44 ± 3 *vs* 42 ± 3) as well as IgA^+ cells (30 ± 3; 34 ± 2; 32 ± 2 *vs* 34 ± 3) were found in the small intestine of non-diabetic NOD mice fed gluten-free, gluten+, and Pregestimil diets compared to the standard diet. Although not significant, slightly higher numbers of CD3- and TCR-$\gamma\delta$-positive cells were recorded in mice on the standard and Pregestimil diets *versus* gluten-free and gluten+ diets.

Discussion

The study provided an unexpected and surprising finding. Gluten-enriched diet, that has been prepared as a modification of diabetes-permissive, non-purified, standard Altromin diet, prevented diabetes in NOD female mice as efficiently (a decrease from 60.6 to 5.9% in diabetes incidence) as did the previously reported [7] gluten-free, non-purified Altromin diet. The gluten+ modification of the standard Altromin diet has been designed in an attempt to prepare a diet that would increase diabetes incidence above the level found with the standard diabetes-permissive diet (and within the same animal facility). No such dietary manipulation of diabetes incidence has so far been reported in the literature.

The gluten+ diet was prepared so that the gluten content in standard Altromin diet was increased on account of meat and corn-starch proteins that have both been repeatedly reported as a non-diabetogenic protein source in non-purified diets [2,3,5,7]. Compared to the standard Altromin diet the gluten-free modified diet also does not contain any corn-starch protein (Table 1). However, studies in both BB rats and NOD mice documented that corn starch is not a diabetes promoting/permissive food component in rodent diets [2–5]. Consistent with previous reports [4,7], the hydrolysed-casein based Pregestimil and the non-purified, gluten-free Altromin control diets again highly prevented diabetes incidence in NOD females (5.9 and 10%, respectively). Insulitis scoring in surviving non-diabetogenic mice revealed a slightly less affected islets in animals fed with the disease-protective diets, although only the effect of gluten-free Altromin diet was statistically significant. Since several animal studies reported the importance of early dietary intervention [4–7], the NOD mice used in this experiment were never exposed (even *in utero*) to other dietary components. The mechanisms by which diets diminish diabetes incidence in NOD mice are not known. Flohé *et al*. [10] reported that wheat-based, diabetes promoting diet induces a Th1 cytokine bias in the gut of NOD mice. Recently, Maurano *et al*. [11] reported signs of enteropathy and increased mucosal levels of inflammatory cytokines in NOD mice fed a standard, diabetogenic, wheat-based diet compared to NOD mice on a gluten-free diet. We found no substantial changes in the subsets of CD3-, TCR-$\gamma\delta$- as well as IgA- and IgM-positive mucosal lymphocytes among the four tested diets. The insulitis scoring as well as the immunohistochemical evaluation of gut lymphocyte subsets were however limited in their sensitivity by the fact that they were carried out on the surviving mice from the diabetes incidence study.

The diabetes-preventive effect of the gluten+ diet reported in this study together with data from literature suggest that dietary factors are not diabetogenic as such but are rather modulating the full penetrance of diabetes in genetically susceptible individuals. Such role of dietary factors would be in accord with the hygiene hypothesis: i.e. lack of induction of regulatory immune responses by environmental stimuli leads to a higher penetrance of the disease in diabetogenic genotypes.

We suggest that two possible mechanisms might be responsible for the dual effect of dietary gluten observed in this study. First, gluten may influence diabetes incidence by a direct effect on the gut mucosa. Gliadin displays lectin-like properties and similarly to LPS directly stimulates innate immune responses through activation of NF-kappaB [12]. High doses or long exposure to LPS induces unresponsiveness or tolerance [13]. Similarly, high doses of gliadin may also have opposite effects at the level of gut mucosa.

Second, mucosal immunoregulation may be mediated via changes in the composition of physiological gut microflora, which is the major maturation force for

DOI: 10.1002/dmrr

the development of gut mucosal immune system. Mucosal surfaces, the major interface between self and the environment, represent a major induction site for regulatory immune responses [14]. In such a scenario one may never find an exact diabetogenic agent or a diabetes-permissive dietary entity. Different food components/compositions that shape gut microflora into a certain profile would lead to diabetes prevention or vice versa. Diets, e.g. elemental diet, have been reported to influence composition of the intestinal microflora [15]. We have recently described a decrease in the number of Gram-positive intestinal bacteria in NOD mice fed the diabetes-preventive, gluten-free Altromin diet [16]. Whether this change in microflora composition is responsible for the diabetes-preventive effect of the gluten-free diet has to be clarified in germ-free NOD mice.

In conclusion, both gluten-free as well as gluten-enriched (gluten+), non-purified diets highly prevented diabetes in NOD mice. We suggest, that dietary modification of diabetes incidence may be mediated by mucosal immune regulation achieved either by direct, innate immune effect of dietary gluten at the level of gut mucosa or by diet-induced changes in the composition of physiological intestinal microflora. Further experiments, including dietary studies on environmentally-defined, gnotobiological germ-free mice are carried out to address the enigmatic effect of dietary gluten in T1D.

Acknowledgements

This study was supported in part by grants from the Danish Diabetes Association (grant no. 21/2005 to D.P.F.), the Hovedstadens Sygehusfællesskab (H/S grant no. 961503094 to K. B.), the Grant Agency of the Czech Academy of Sciences (grant no. IAA5020405), the Grant Agency of the Czech Republic (grant. no. 303/061329), and by the Institutional Research Concept AV0Z50200510 (Institute of Microbiology, Czech Acad. Sci.). We thank J. Pedersen, P. Albrectsen, D. Borovská, and M. Baeksted for their excellent technical assistance.

Conflict of interest

None declared.

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