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Molecular characterisation of candidate wheat proteins associated with the development of type 1 diabetes

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Thesis submitted to the Department of Biochemistry, Microbiology and Immunology in partial fulfilment of the requirements for the Ph.D. degree

University of Ottawa
Ottawa, Ontario, Canada
January 18, 2002

ABSTRACT

A wheat gluten (WG) diet promotes the development of type 1 diabetes (T1D) in diabetes prone BioBreeding (BBdp) rats. Our aim was to examine whether oral exposure of neonates to WG proteins prior to weaning would modify the disease process and to identify candidate wheat proteins that may be related to development of T1D. Feeding diabetes-promoting WG proteins during the first week of life protected a proportion (~1/2) of animals from developing diabetes as adults. Twenty-four hours after exposure to a cereal-based diet there was a downregulation of the Th1 cytokine gene IFN-γ in the small intestine. A cross-sectional analysis of 1D-Western blots of WG proteins showed that 70 d BBdp rats have higher IgG reactivity to 33 and 36 kDa WG proteins than diabetes resistant BB rats. Similarly, prospective analysis of pre-diabetic and asymptomatic BBdp rats showed that increased reactivity to 33 and 38 kDa WG proteins was associated with progression to disease. We detected increased IgG reactivity to 33 and 38 kDa WG proteins in Finnish children newly diagnosed with diabetes compared with age, sex and HLA-DQ matched children without diabetes. 2D-Western blots showed that 11 wheat proteins were antigenic in the majority of BBdp rats. These 11 proteins were analysed by mass spectrometry and searches of amino acid sequences in public databases suggested that two candidate wheat proteins were alanine aminotransferase 2 transaminase 2 (GPT-2, TC# 8197) and wheat storage globulin (WSG, Acc.No.AAA3469). GPT-2 and WSG have similar amino acid sequences as human heat shock protein 60 (HSP60), an autoantigen in T1D. Moreover, WSG shares sequence homology with the insulin receptor precursor, interleukin-1 (IL-1) receptor and IL-1 beta. These structural homologies to diabetes related antigens suggest that wheat proteins may

be involved in the autoimmune process that destroys β-cells. As part of a strategy to develop assays of food protein "diabetogenicity", we showed a major down regulation in protein expression in gut epithelial cells exposed to chymotrypsin digested WG proteins. These studies are the first direct molecular characterisation of diabetes related dietary wheat proteins that may be implicated in the development of T1D.

DEDICATIONS

To my parents, Tadeusz and Krystyna, and to my grandfather Sebastian, who have taught me to pursue excellence, to embrace challenges and to believe in my dreams

ACKNOWLEDGEMENTS

Thank you to my mentor and best teacher Dr Fraser Scott, who exemplifies excellence, perseverance and strength. I would like to acknowledge the guidance and helpfulness of professors and support staff from the department of Biochemistry, Microbiology and Immunology of the University of Ottawa, as well as from the Ottawa Health Research Institute. Thank you to everyone at the Nutrition Research Division at Health Canada for encouraging and funding my research. I would like to thank our collaborators, Drs John Kelly, Pierre Thibault, Hubert Kolb, Stefanie Flohé, Olli Simell and Tuula Simell for their help and contributions. Many thanks for the technical support provided by Jocelyn Souligny, Dominique Patry, Heidi Gruber, and Penny Jee. I would like to acknowledge the funding provided by the Juvenile Diabetes Foundation International, the Canadian Foundation for Innovation and the Ontario Research and Development Challenge Fund. I am grateful to the Diabetic Children's Foundation and the Canadian Institutes of Health Research for supporting my studies through the Doctoral Research Award.

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ABBREVIATIONS

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2D-PAGE	Two dimensional polyacrylamide gel electrophoresis
AIN-93G	rodent diet recommended by the American Institute of Nutrition
BBc	BioBreeding non-diabetes prone control rat
BBdp	BioBreeding diabetes prone rat
CD	celiac disease
ECL	enhanced chemiluminescence
GAD	glutamic acid decarboxylase
GALT	gut associated lymphoid tissue
GB-1	globulin Beg1 precursor, barley
GPT-2	alanine aminotransferase-2 (glutamic pyruvic transaminase)
HC	hydrolysed casein
HLA	human leukocyte antigen
HMM	high molecular mass
HSP 60	heat shock protein 60
IEF	isoelectric focussing
IEC-6	rat intestinal epithelial cell line
LMM	low molecular mass
MS/MS	tandem mass spectrometry
NCBI	National Center for Biotechnology Information
NIH-07	Defined cereal-based rat diet from the National Toxicology Program, NIH
NOD	non-obese diabetic mouse
PG	Pregestimil®, hydrolysed casein-based formula
pI	Isoeletric point
ROC plot	Receiver Operation Characteristic plot
RT-PCR	Reverse transcriptase, polymerase chain reaction
SPI	soy protein isolate
Th	T helper cell
TID	type 1 diabetes
WG	wheat gluten
WSG	wheat storage globulin

INTRODUCTION

The combination lock

Type 1, insulin dependent diabetes (T1D)[1], is an organ specific autoimmune disease characterised by spontaneous, immune-mediated destruction of insulin-producing β -cells in the islets of Langerhans in the pancreas. In humans, the development of the disease generally takes several years. T1D has been classified as an autoimmune disease because the immune system fails to maintain tolerance to pancreatic β-cells and targets these insulin-producing cells for destruction [2]. Neither the instigating event(s), or what regulates the pace of T1D progression is known. At least 20 autoantigens have been identified, the ones recognised in the early stages of the disease are glutamic acid decarboxylase (GAD 65), insulin and islet cell antigen 512 (ICA512) [3]. This list of target antigens is neither exhaustive nor complete. There may be other (auto)antigens that participate at earlier stages of the immune process to destroy β-cells. A major issue is the molecular identity of these candidate antigens and their role in the pathogenesis.

T1D has been described as a T cell mediated disease because of the presence of activated T cells in islet lesions of newly diagnosed T1D patients [4]. Healthy individuals harbour auto-reactive T cells but their activity is regulated to prevent abnormal reactions against self-proteins. Most auto-reactive T cells are deleted during negative selection in the thymus and those that escape are regulated through anergy, inhibition or suppression [2]. Autoreactive T cells do accumulate in the pancreas and it was reported that a minimum number must be present before an environmental agent will prompt the development of T1D [5]. These T cells may have an important effector function in the

[1] The nomenclature has been changed from insulin dependent mellitus (IDDM) to T1D [1]

development of the disease. However, the series of events that initiate and perpetuate the immune attack that culminates in the destruction of β-cells have not been elucidated.

The pathogenesis of T1D progresses in stages. The appearance of peri-insulitis, characterised by an accumulation of antigen presenting cells (macrophages and dendritic cells) as well as B and T lymphocytes in the area outside of the islet, occurs in the first phase of the disease. This is followed by infiltration of the islets, intra-insulitis, and lymphocyte mediated destruction of the β-cells. In the last stage of the disease, once the majority of the β-cells have been destroyed, the patient develops clinical symptoms of T1D.

Onset of T1D is acute and may occur at any age, although it is rare in the first year of life. The incidence is highest in children of 10-14 years of age, however more than 50% of all T1D develops after the age of 20 [6]. The loss of β-cells is permanent, consequently the patient becomes dependent on exogenous insulin. Although insulin delivery allows the individual to thrive, these patients are nevertheless at increased risk for complications later in life such as blindness, vascular disease, nephropathy, neuropathy and early mortality.

T1D is a heterogeneous and polygenic disease that develops as a result of an interaction among several risk genes [7, 8] and environmental stimuli [9]. Genes encoding molecules associated with antigen presentation to T lymphocytes, principally HLA-DR3 and DR4, as well as DQβ-chain account for 45% of genetic susceptibility [10]. However, T1D cannot be predicted based solely on genetic markers as shown in genetically identical, monozygotic twins, which are concordant for diabetes in only one third of the pairs, and 12% in dizygotic twins [11]. This illustrates the multifactorial

nature of T1D and emphasises the influence of environmental factors in modifying the development of the disease.

Over the last two decades the incidence of T1D has been increasing worldwide and particularly in Western countries [12, 13]. Because improved survival rates for persons with diabetes cannot account for the increased number of patients with T1D, particularly as 90% of new cases do not have a first-degree relative with diabetes, these observations imply that agents in the environment are important in the pathogenesis of the disease. Moreover, the incidence of T1D varies greatly among countries, from highest in Finland, where the reported incidence is 42.8/100 000 person·year, to the lowest in Japan, where it is 0.6/100 000 person·year [14]. Most notably, immigrant populations adopt the incidence of diabetes of their new country [15].

The aetiology of T1D is complex because it requires an interaction between multiple genes and environmental factors. Three major environmental factors have been the central focus: viral agents (i.e. Coxsackie virus and cytomegalovirus), early infant diet (i.e. breast-feeding, cow milk) and β-cell cytotoxins (N-nitroso derivatives) [3]. The network of genetic and environmental factors work in combination, much like the tumblers of a lock to modify the immune homeostasis in the pancreas [16]. It is unlikely that there exists a single combination for all individuals that will lead to the development of T1D because of the genetic, biochemical and immunological diversity of humans and the heterogeneity in the type and quantity of environmental exposures. However, in this complex network of diabetogenic factors there are interventions that modify the course of pre-clinical diabetes and supersede the influences of other tumblers in the combination.

The pancreas

Regardless of which determinants contribute to the immune mediated attack, the target is always a single cell type in the endocrine pancreas, the insulin producing β-cell in the islets of Langerhans. Even though the islets consist of multiple cell types, α-cells (glucagon), δ-cells (somatostatin) and PP cells (pancreatic polypeptide), these cells are not part of the autoimmune reaction. Likewise, the exocrine pancreas, consisting of the acinar cells, which secrete proteolytic enzymes (trypsin, chymotrypsin, carboxypolypeptidase, ribonuclease and deoxyribonuclease), a carbohydrate enzyme (pancreatic amylase) and a lipid enzyme (pancreatic lipase) are believed to be normal in patients with T1D. And so despite the presence of other cells in the vicinity, the β-cell appears uniquely sensitive to various environmental and genetic factors.

Rodent models of autoimmune T1D

Animal models of T1D provide a useful surrogate population to study the contribution of individual determinants (or tumblers) in T1D progression in an isolated and controlled environment. There are two rodent models of spontaneous immune-mediated diabetes that resemble closely the clinical disease in humans, non-obese diabetic (NOD) mice and diabetes-prone Bio Breeding (BBdp) rats. NOD mice and BBdp rats both exhibit an aggressive immune attack on their beta-cells, develop insulitis, and present with the same clinical symptoms as human patients; polyuria, polydipsia, weight loss and hyperglycemia. Although both rodents are recognised as good models of the human disease, there are some immunological differences between the rodent models and humans. For instance, NOD mice have increased numbers of T lymphocytes in several tissues, while BBdp rats are T-cell lymphopenic. NOD mice also show some abnormalities in antigen presenting cells [17], whereas BBdp rats do not exhibit normal

cytotoxic T-lymphocyte activity [18]. There are also some notable differences in the progression of the disease between NOD mice and human patients. First, female NOD mice exhibit higher incidence of T1D (>90%), while the incidence in male mice is generally <40% [17]. In humans, T1D occurs equally in both sexes in patients under 15 years of age. Second, the age at onset in NOD mice occurs typically in adulthood at 20-30 weeks, which is later than in human patients, in whom the mean age of onset is usually in adolescence. The inflammatory process in the pancreas of BB rats follows more closely the histological picture seen in humans. The incidence of disease in male and female rats is similar (reviewed in [19]) and clinical onset is abrupt, occurring usually during adolescence with a mean age at onset at around 90 days. Progression to diabetes in these rodents is marked by MHC class 1 hyperexpression in the islets before classic light-microscopy-defined infiltration of mononuclear cells [20]. The immune destruction of the islets progresses at various rates culminating in diabetes, similar to human T1D. Our studies used the Ottawa colony of BB rats to examine the role of environmental agents in T1D progression.

Viral etiology

Case reports of viral infections preceding the onset of T1D provide anecdotal evidence for the association of viruses in the pathogenesis of the disease. Several candidate viruses have been implicated, such as mumps, rubella, Coxsackie virus B4, cytomegalovirus, polio, measles, influenza and tick-born encephalitis [21]. One of the more striking reports was the study of children with congenital rubella syndrome in New York, NY, where approximately 20% of these children developed diabetes [21-23]. More recently, Juhela *et al.* reported that children diagnosed with diabetes in the

preceding 4-72 months had increased T-cell responses to Coxsackie virus B4 proteins compared with healthy control children [24]. However, many infants come in contact with enteroviruses [25, 26], which illustrates the difficulty in identifying factors that are commonly encountered in the environment but that may be handled differently by people at risk of T1D. The most persuasive examples that certain viruses influence diabetes outcome have been shown in animal models of T1D [22, 27]. A viral aetiology for T1D is supported by the presence of homologous sequences between Coxsackie virus P2C peptides and one of the major autoantigens in T1D, glutamic acid decarboxylase (GAD65). However, despite these sequence similarities, Schloot *et al.* showed that T cell clones specific for GAD65 isolated from patients with T1D had sequence homology to Coxsackie virus P2C peptides but did not cross-react with the viral peptides [28]. Moreover, exposure to infectious agents appears not to be a prerequisite for the development of diabetes in diabetes-prone BioBreeding (BBdp) rats and non-obese-diabetic (NOD) mice because a high incidence of T1D is observed in ultra clean (viral-antibody-free) conditions [29, 30]. Although in humans, some epidemiological studies suggest a temporal association between infectious agents and a rise in the expression of T1D [31, 32], there is no conclusive evidence for the involvement of viruses in the pathogenesis of this disease. Some studies of pancreata of patients with T1D probed for viral nucleic acid sequences using PCR and they did not detect potentially diabetogenic viral sequences [33, 34]. Other retrospective analyses examined the association of enteroviral infections and T1D and concluded that individuals from populations with a high incidence of disease, such as Finns, did not have high levels of antibodies to enteroviral proteins, nor did they have elevated frequency of enteroviral infections [35].

Similarly, a study of the immunisation records in high-risk populations did not show any temporal association with T1D [36]. Although viral aetiology is an attractive explanation for the development of T1D, a causal association remains to be shown. The difficulty in linking viral infections with diabetes highlights the complexity associated with identifying factors that are commonly encountered in the environment but that may be handled differently by people at risk of T1D. Added to this complex aetiology are dietary agents that may also modify the course of T1D.

Diet and type 1 diabetes

Characterisation of the cells that participate in the immune response in the pancreas suggested that T1D is a Th1-cell mediated disease [37]. Inflammation in the pancreas of BB rats and NOD mice is associated with T helper cells secreting type 1 cytokines (Th1), IFN-γ and TNF-α [30, 38]. Diet was shown to shift the cytokine profile in the pancreas from a type 1 toward a type 2 (Th2) predominance, characterised by TGF-β, IL-10 and IL-4 [30]. For example, a hydrolysed casein-based (HC), diabetes retardant diet was associated with a shift in the predominance of Th1 to Th2 cells in the pancreas. From this we can infer that information about luminal antigens is conveyed to the pancreas and has the potential to alter the immune homeostasis.

T1D is diet-induced in 50% or more of cases in both animals models, BB rats [39, 40] and NOD mice [41, 42]. In particular, cereal based diets, such as the standard open-formula rodent diet NIH-07, consisting of 82% plant materials are associated with a high incidence of T1D in rodents [40, 43]. Semi-purified diets consisting of casein, hydrolysed casein or an amino acid mixture protected the animals from the development of T1D [44]. Plant proteins such as wheat gluten (WG) and soy were associated with the highest

diabetes inducing potential when fed to BBdp rats [39, 40, 45] and NOD mice [42, 46]. Scott *et al.* showed that delaying the introduction of a diabetogenic diet until 50 days old delayed the onset of diabetes [30, 40]. Furthermore, they reported that there was a window of time when the development of diabetes could be modified. Feeding BBdp animals an HC diet from day 23-50 and then switching to an NIH diet, delayed the onset of T1D by the same number of days, but resulted in the same incidence as animals that were fed NIH exclusively. In contrast, late exposure to NIH, whereby animals were fed HC from day 23 until 100 and then switched to NIH, induced diabetes at a similar rate as animals fed HC only. Early, but transient exposure to dietary diabetogens in childhood, as in the group of rats fed NIH from day 23 till day 50 and then switched to HC, showed that the rate of disease progression and the incidence were akin to the trend observed when animals were fed HC exclusively. These findings suggested that the progression to T1D can be modified by the diet fed even as late as puberty, from which point onwards, long-term, constant exposure to a cereal based diet promoted the development of the disease. The data imply that these dietary agents modify disease progression by a sustained stimulation of the immune system rather than a single event that triggers the autoimmune response.

Diet was shown to modify islet morphology before insulitis is visible in the islets by light microscopy. BBdp rats fed the diabetes-retardant HC diet had 65% greater total islet area compared with rodents fed the diabetes-promoting NIH-07 diet [30]. Moreover, the HC diet maintained the same islet area in BBdp as in BBc rats. These findings suggest that dietary proteins not only evoke a beta-cell specific immune response but also may have direct consequences on islet homeostasis.

Dietary antigens that come in contact with the gut associated lymphoid tissue (GALT) can induce systemic responses, either oral tolerance or immune priming. The immune response to most dietary antigens is oral tolerance, whereby there is hyporesponsiveness of mature lymphocytes in peripheral lymphoid tissue upon subsequent challenge with the relevant antigen. The major regulatory mechanisms that establish and maintain tolerance are clonal anergy (the lack of co-stimulatory molecules), clonal deletion and activation of regulatory $CD4^+$ T cells that suppress other immune reactions via the production of inhibitory cytokines such as IL-10 and TGF-β . This state of hyporesponsiveness depends on the nature of the antigen and dose, as well as the state of the host immune system.

Cow's milk is suspected to be a diabetogenic food source in susceptible individuals [12]. The major antigenic proteins in cow's milk were reported to be bovine serum albumin (BSA) and a 17 amino acid albumin peptide (pre-BSA from position 152 to position 169, ABBOS) [47]. Because antibodies to the ABBOS peptide were shown to precipitate an islet protein, ICAp69 [48], it was hypothesised by Dosch *et al.* that the cross-reactive β-cell p69 antigen perpetuates the antibody response until the destruction of islet β-cells is complete. A subsequent study by Atkinson and colleagues [49] did not find an increased level of anti-BSA antibodies in patients with recent onset T1D than in control subjects. In addition, Saukkonen *et al.* [50] could not detect cross-reactivity between BSA and β-cell surface proteins. Although, the diabetogenic potential of milk remains controversial, nevertheless this concept has important implications, especially with respect to early exposure of infants to cow's milk and warrants further investigation.

The only prospective nutritional intervention trial in humans is the Trial to Reduce IDDM (insulin dependent diabetes mellitus) in the Genetically at Risk (TRIGR). The aim of the trial is to evaluate whether avoidance of intact cow's milk proteins during the first 6 months of life would inhibit the development of T1D in children with HLA alleles associated with diabetes or first-degree relatives of people with diabetes [51]. The data suggest that high risk Finnish infants who avoided cow's milk and were fed a hydrolysed casein based infant formula or breast-fed for the first 6-8 months of life exhibited fewer islet autoantibodies [52]. It was reported that both cellular and humoral responses to bovine insulin were increased in the group of 3-month-old infants fed cow milk formula as compared to infants breast-fed exclusively. These results suggest that there is an active exchange of potentially diabetogenic signals between the gut, its contents and the pancreas. By corollary, these findings also imply that there exists a potential window of time during infancy when dietary intervention can modulate immune responses in humans at risk of T1D toward a non-destructive pathway.

Feeding studies in the BBdp rat showed that a diet consisting of cow's milk proteins induced a smaller and more variable incidence of T1D than a diet of wheat gluten and soybean proteins [40]. Soy proteins were studied using 1D Western blots to characterise the immunoreactive proteins in susceptible individuals. The results suggested that the diabetogenic components in soy may be associated with the 11S glycinin subunit [53]. Less information is available about the immunoreactivity of wheat gluten proteins in patients with T1D or in animal models of the disease.

The chemistry of wheat gluten proteins

Crushing grains of wheat (*Triticum aestivum*) into flour and kneading the paste under a small stream of water to remove starch and most albumin/globulin proteins produces a cohesive dough consisting mainly of wheat gluten proteins (WG). Wheat proteins were originally classified according to their solubility in water (albumins), dilute salt (globulins), dilute alcohol (gliadins) and dilute acetic acid (glutenin) [54]. However, each of these fractions is a complex mixture of proteins with overlapping solubility. An alternative classification was proposed by Shewry [55], which was based on amino acid composition and chemical structure. The majority of proteins in WG are gliadins and glutenins, which are storage proteins from wheat endosperm that provide nitrogen to the germinating cereal seed embryo and so they tend to be rich in asparagine, glutamine, arginine or proline [56]. Gliadins can be divided into four groups, called α-, β-, γ- and σ-gliadins based on their electrophoretic mobility in acidic pH [57]. Some gliadins are monomers that are linked by intra-chain disulphide bonds (α-, β- and γ-gliadins), while others possess no disulphide bonds (σ-gliadins). In contrast, glutenin proteins exhibit both inter- and intra-chain disulphide bonds, which facilitates the crosslinking of multiple small polymers into some of the largest protein structures in nature [58]. Once the disulphide bonds are reduced, low (LMM) and high molecular mass (HMM) glutenin proteins can be separated by sodium-dodecyl-sulphate polyacrylamide electrophoresis (SDS-PAGE) [56]. LMM glutenins resemble α-gliadins in amino acid composition, while HMM glutenins have fewer prolines [56].

Wheat grains are synonymous with good quality nutrition and wheat flour is a staple food in much of the world. Although, the consumption of wheat has been increasing on average 1% per year over the last 20 years [59], the nutritional value is

actually poor due to the low total protein content and paucity in essential amino acids [60]. Although wheat grains are harmless in healthy individuals, they can induce primary and secondary intolerance in some individuals, such that they are either not tolerated or toxic when ingested. A case in point is the patient with celiac disease.

Celiac disease and type 1 diabetes

Gluten-sensitive enteropathy (celiac disease, CD) is an example of a food-induced immune-mediated disorder in which ingestion of cereal grains, or more specifically gliadin proteins induces a T cell mediated attack on the gastrointestinal tract [61]. Although gliadin-specific, DQ2-restricted T cells have been reported in the gut mucosa of CD patients [62], the pathogenesis of CD has not been resolved. Several mechanism(s) have been proposed: 1) a missing enzyme, 2) an immunological reaction, 3) a membrane glycoprotein defect (gluten-lectin) and/or 4) a defect in the permeability of the gut mucosa [60]. The favoured hypothesis suggests that alpha gliadin-derived peptides are presented on disease associated DQ2 and DQ8 molecules that are recognised by aberrant mucosal T cells [63]. Recognition of the offending proteins produces an inflammatory immune response that results in intestinal damage. Recently, it has been reported that host tissue transglutaminase deamidates glutamine residue 65 of an alpha-gliadin peptide, which produces an immunodominant CD-specific T-cell epitope [64].

Patients with CD often present with malabsorption, diarrhoea, vomiting and failure to thrive, but some individuals are virtually asymptomatic or have a silent form of CD. The prevalence of latent or silent CD varies worldwide [65-69]. Many patients with CD remain undiagnosed because they show no clinical symptoms of the disease. In Italy, for example, the ratio of known to undiagnosed CD cases was estimated to be 1 to 7 [70].

Prolonged dietary exposure to wheat gluten proteins among patients with CD has been correlated with increased risk of autoimmune disorders [71]. For example, as many as 10% of diabetic patients also have autoimmunity typical of CD, a rate that is 17-33 times that of the general population [72]. Interestingly, when CD and TID are present in the same individual, diabetes develops less frequently after the diagnosis of celiac disease. This observation could be associated with the protective effect of a gluten-free diet (used to treat CD patients) in type 1 diabetic patients [73]. One explanation for the link between CD and T1D may be genetic, since they are associated with human leukocyte antigen (HLA)-DQ2 (DQA1*0501-DQB1*02) and DQ8 (DQA1*0301-DQB1*0302) [10, 74, 75]. Therefore, the coexistence of these diseases in some individuals may be attributed to the presentation of similar antigen epitopes.

The immunology of food

The gut immune system is a major site of exposure to environmental agents. It is continuously challenged by a wide spectrum of dietary macromolecules, their metabolic by-products, resident bacteria and invading viruses. Thus, food is a source of sustenance as well as information about our environment. The potential of diet to modulate immune reactions is well documented. Li *et al.* reported hyperexpression of MHC class 1 molecules on the surface of the β-cell in association with insulitis and diabetes in BBdp rats fed a cereal-based rodent diet (Purina 5001) [20]. Another study noted that animals fed the HC diet had a significantly lower number of macrophages in their spleen, an increased number of total T cells in the mesenteric lymph nodes, and their natural killer cells exhibited lower cytotoxicity than animals fed a cereal-based diet [76]. Others examined the effect of a WG diet on the immune status of patients with autoimmune

T1D. Tommasini *et al.* reported that anti-pancreas antibodies disappeared in patients with T1D on a gluten-free diet, suggesting that anti-pancreas autoimmunity may be gluten dependent [77]. Functional studies measuring T cell proliferation in the presence of enzymatically hydrolysed WG proteins found cell-mediated immunity against gluten in newly diagnosed patients with T1D, although the level of stimulation was low [78]. There are many questions related to the identity of the wheat proteins that are immunogenic in diabetes-prone individuals and to the pathways that promote an autoimmune reaction in response to wheat gluten ingestion.

Hypothesis

The essential question with regard to the initial events in the WG-related diabetes is the identity of the wheat proteins that participate in the progression of the disease. The leading theories propose three putative pathways: 1) molecular mimicry; 2) cytokine profiles modified by diet (bystander activation); and/or 3) dietary modulation of β-cell growth and metabolism. These processes are not mutually exclusive and one or all may be active at different stages in the pathogenesis of T1D.

Ingestion of wheat proteins may promote a pro-inflammatory response in the gut epithelium in susceptible individuals, which may damage the gut barrier, increasing permeability to luminal antigens, making it difficult to induce normal oral tolerance. In diabetes-prone rats fed a diabetogenic diet, there may be a breakdown in the tolerogenic mechanisms that normally prevent Th1 responses to dietary proteins. The immune response may be a result of changes to immune regulation and/or barrier function/permeability. The net effect is IFN-γ dependent gut inflammation with sensitisation of $\alpha_4\beta_7^+$ mucosa/pancreas seeking T cells and extension of inflammation to

the pancreas. Inflammation in the pancreas may expose islet cell autoantigens, some of which may share structural homology with wheat proteins. Repeated exposure to wheat proteins in the diet may perpetuate an immune reaction against islet autoantigens by molecular mimicry, leading to insulitis and β-cell destruction in a diabetes-prone genetic background. As many as 10% of gut lymphocytes normally travel from the gut immune tissues through the pancreas. Among the T cell repertoire of diabetes prone individuals, there are β-cell reactive T cells that could be activated either non-specifically at a site of chronic inflammation (molecular mimicry) or following exposure to antigenic sequences or immune mediators from the gut lumen (bystander activation).

We posit that the gut has an essential role in the pathogenesis of T1D in BB rats and NOD mice, possibly as a source of inflammatory cells that travel to the pancreas and attack insulin-secreting β-cells by molecular mimicry and also as source of activated immune cells that secrete inflammatory cytokines (Il-1) that could damage β-cells as innocent bystanders. An essential feature of this hypothesis is the identity of the agents in wheat that stimulate an immune response in individuals that develop T1D. We will be able to better speculate about the mechanism(s) that lead to this autoreactivity once we have precise information on the molecular identity of the diabetogenic agents in wheat. The focus of this project was to identify diabetes-related wheat proteins.

Questions and Methodology

Certain wheat proteins may be interpreted differently by the gut immune system of susceptible individuals resulting in changes in immune regulation and/or barrier permeability. The objective of this research was to identify and characterise wheat gluten proteins that are uniquely immunogenic in animals at risk of diabetes or in patients with

diabetes using proteomic analyses, immunoblotting and mass spectrometry. We used Western immunoblots of one- and two-dimensional gels to examine antibody reactivity of more than 200 WG proteins in serum from 1) BB rats at different risk for development of diabetes: BBc (non-diabetes-prone control), BBdp (diabetes-prone) and BBd (overt diabetic) and 2) Finnish children newly diagnosed with diabetes. The proteins of interest were analysed by mass spectrometry to determine their amino acid sequences. These amino acid sequences were compared with sequences in the NCBI database [79] to identify homologous proteins. The major research questions addressed in this study were as follows:

1. Does exposure to WG proteins in the neonate modify the development of T1D?
2. Do susceptible individuals show different (diabetes-related) antibody reactivity to wheat gluten? Can this immunoreactivity be used to identify specific molecular sequences of diabetes-related peptides or proteins?
3. Are there sequence similarities between candidate wheat proteins and diabetes related antigens?

Although T1D has been suggested to be mediated by autoreactive T cells, a recent report suggests that auto-specific T cells alone are insufficient to induce autoimmunity [80]. Their findings show that chronic inflammation and stimulation of T cells in lymph nodes are the essential prerequisites for progression of the disease. In our studies we have used antibodies as surrogate markers of an abnormal immune response against certain WG epitopes that could reflect abnormal T cell reactivity or regulation.

Similar studies of human using serum antibodies from patients diagnosed with Stiff-man syndrome and T1D were used to characterise the 64 kDa antigen in both BB

rats and human islets [81, 82]. Moreover, 2D gel analyses of immunoprecipitated human and rat islet proteins showed that the 64kDa antigen was specifically and consistently recognised by autoantibodies in sera from patients [83]. This antigen was subsequently identified as glutamic acid decarboxylase (GAD), one of the major islet cell autoantigens in T1D [84]. It was later reported that antibodies to GAD may be an early marker of β-cell autoimmunity and can be used to screen for risk of the development of T1D [85]. However, there is debate whether GAD antibodies are serological markers that are closely correlated with cellular autoimmunity [86, 87]. Most likely, patients with T1D have both humoral and cellular reactivity to GAD, but the immune responses can be detected at different times in the course of the disease. Although, it is not clear how the antibodies are related to the pathogenesis of T1D, the association of GAD to T1D has lead to successful prophylactic interventions in animals [88, 89] as well as to development of novel therapies of T1D [90, 91].

Others have studied antibody responses to food antigens to identify inappropriate immunological responses and gut hypersensitivity disorders [92, 93]. Western immunoblotting was used to characterise allergens in fruit [94] and cereals [95, 96] as well as autoantibodies in rheumatic disease [97, 98], systemic lupus erythematosus [99] and diabetes [100]. There are reports of a transitory rise in anti-gliadin antibodies in newly diagnosed patients with T1D [101, 102]. Likewise, the relationship between CD with T1D has been investigated using serological tests for anti-endomysial and anti-gliadin antibodies [103, 104]. However, the humoral reactivity to other wheat gluten proteins has not been examined in patients with T1D. The molecular characterisation of WG antigens may identify potential surrogate markers of T1D. Our findings may also be

applied to the development of a therapy for modulating immune responses in the gut of susceptible individuals.

Our aim was to identify the diabetes-related wheat proteins in newly diabetic patients and pre-diabetic animals by studying the IgG reactivity to wheat proteins. This was a novel approach in the study of diabetes-related dietary proteins. We combined the resolving power of 2D gels with the sensitivity of Western immunoblots to pinpoint antigenic proteins using antibodies from overt diabetic or diabetes-prone rats and humans. Mass spectrometric analyses of proteins of interest yielded an amino acid sequence that was used to find homologous proteins. We have also applied this proteomic technology to devise an *in vitro* test system to examine the effect of dietary diabetes-promoting proteins on gut intestinal cells, which may be involved in the diabetes process. Others have used a similar *in vitro* test system in combination with proteomic tools to study β–cell maturation and response to chemical mediators [105]. Elucidating the chemical nature of antigenic wheat proteins in diabetes-prone individuals will facilitate future studies of the association between the gut and the pancreas in the pathogenesis of T1D.

MATERIALS AND METHODS

Patients

Serum samples from Finnish children newly diagnosed with diabetes (n= 23; average age 9.8 $\pm$ 3.4 yr) and non-diabetic control children (n=37; average age 9.9 $\pm$ 3.5 yr), matched for age, sex and HLA-DQ antigens, were provided by Drs Olli and Tuula Simell (Department of Paediatrics, University of Turku, Turku, Finland).

BB rat husbandry and diabetes monitoring

BioBreeding [106] (BB) control, (non-diabetes-prone, BBc) and diabetes-prone (BBdp) rats were obtained from the Animal Resources Division of Health Canada where they were maintained under specific pathogen free conditions. The colony is antibody-free with respect to Sendai virus, Pneumonia virus of mice, Rat Caronavirus/Sialodacryoadentis virus, Kilham rat virus, Toolan's H-1 virus, Reovirus type 3, *mycoplasma pulmonis*, Lymphocytic choriomeningitus virus, Mouse polio virus, Encephalitozoon cuniculi, Hanta virus, CAR bacillus, rat parvovirus, NS-1. Animals were housed in banks of 30 stainless steel, wire-bottom cages, and they were permitted free access to food and water. Glucose was measured in urine (Testape, Lily, Toronto, Ontario) twice each week starting at 55 days of age. When the value exceeded 2, animals were fasted overnight, a drop of blood was obtained from the tail vein and glucose was measured with a glucometer. Animals with blood glucose $\geq$ 200 mg/dl (11.1 mM) were killed by exsanguination while under 3% isoflurane or halothane in oxygen anaesthesia. The significance of differences among diet treatments for final diabetes incidence was evaluated using Fisher's exact test (2-tailed). Each group consisted of 15-17 animals. Animals diagnosed with T1D were killed and the results are reported as the percent of animals without T1D (survival graph).

Early oral feeding

In order to examine the effect of early oral exposure to WG proteins, a protocol used previously [107] was employed. In brief, neonatal BB rat pups were removed from their dams, fasted for one hour and hand fed once daily from 4 to 7 days old a mixture of Pregestimil® (Mead Johnson & Company, Belleville, Ontario), a hydrolysed casein based infant formula that contains hydrolysed casein plus wheat gluten proteins (ICN) or ground NIH-07 diet (NIH; Ziegler Brothers, Gardner, PA), or Pregestimil® alone. Pregestimil® contains 13% hydrolysed casein, 62% carbohydrates (corn starch and corn syrup solids), modified coconut oil, supplemented with vitamins A, B_1, B_2, B_3, B_6, B_{12}, C, D, E, K, biotin, folic acid, α-pantothenic acid, the amino acids L-tryptophan, L-cystine and L-tyrosine, as well as mineral supplements. Animals were fed 1 mg protein per gram body weight of the pups at the time of feeding, a dose known to induce oral tolerance [108]. WG was given at a dose of ~20 mg/0.45 ml. NIH-07 was given at a dose of 67 mg/0.45 ml. The pups were fed through Technicon-B rubber tubing attached to a 23G needle and a one ml syringe. The diet mixture was kept at body temperature during feeding. After feeding, the pups were returned to their dams and the pups remained with the dams until weaning at 23 days. The animals were weaned onto NIH-07, or one of the semi-purified diets in which HC or WG was the sole source of amino acids. Animals diagnosed with T1D were killed and the results are reported in a survival graph showing the percent of animals without T1D (survival compared by Kaplan-Meier, log rank test).

Diets

BB rats were weaned at day 23 onto a designated diet. The positive control, diabetes-inducing diet, was a standard open formula, cereal-based laboratory rodent diet called NIH-07 (NIH; Ziegler Brothers, Gardner, PA). The NIH diet consisted of 5% dried

skim milk, 10% fish meal, 12% soybean meal, 4% alfalfa, 3% corn gluten, 24.5% ground corn, 23% ground hard winter wheat, 10% wheat middlings, 2% Brewer's yeast, 1.5% molasses, 2.5% soybean oil and vitamins [109].

The diabetes-retardant diet was a modification of the American Institute of Nutrition recommended diet for rats and mice (AIN-93G) in which the sole source of amino acids was hydrolysed casein (HC; Pancase S, Redstar Bioproducts, Tara, Ontario). The HC diet contained 20% hydrolysed casein, 53% corn starch, 5% cellulose-type fiber (Solka-Floc), supplemented with 3.5% AIN-93G mineral mix (ICN Biochemicals, Cleveland, Ohio) and 1% AIN-93G vitamin mix (ICN Biochemicals).

The WG diet, a modified AIN-93G semi-purified diet, was composed of 22.5% untreated wheat gluten (ICN 101815), 50.2% corn starch, 12% sucrose, 5% corn oil, 5% alphacel (cellulose-type fibre), 3.5% AIN-76 mineral mix (ICN Biochemicals, Cleveland, Ohio), 1% AIN-93G vitamin mix, 0.2% choline bitartate, 0.02% DL-methionine, 0.5% L-lysine, 0.08% L-threonine.

Gut cytokines

Cytokine profiles were measured using Reverse transcriptase and polymerase chain reaction (RT-PCR) in samples from pups fed on day 5 as described in section "*early oral feeding*" (page 19) and euthanized 24 hours later. Total RNA was isolated from the duodenal section of the small intestine using Trizol Reagent (Life Technologies, Karlsruhe, Germany) as per manufacturer's recommendations. RT-PCR was performed as previously reported [110]. Primer sequences were purchased from Clontech (β-actin, IFN-γ, TGF-β, Palo Alto, CA). The products from 22 PCR cycles for β-actin and 34 PCR cycles for IFN-γ and TGF-β were within the semi-quantitative range as verified by

initially performing serial dilution of cDNA and varying the number of cycles used for PCR. Amplified products were visualised in a 1.5% agarose gel containing ethidium bromide. The fluorescence intensity of each band was measured as Boehringer Light Units (BLU) using a Lumi-Imager™ (Boehringer Mannheim, Mannheim, Germany) and the specific analysis software (LumiAnalyst™ 3.x). The amount of cDNA was plotted versus the corresponding BLU to generate a curve to which linear regression was applied. The mRNA amount was set as the BLU value for 1 µl cDNA. The correlation between different amounts of cDNA and the corresponding fluorescence intensity ranged between 0.90 and 0.99. The relative amounts of cytokine mRNA were expressed as arbitrary units as the ratio of cytokine to the respective β-actin mRNA.

Immunoblotting of protein extracts of wheat gluten

Wheat gluten proteins were extracted from wheat gluten powder (ICN Biochemicals, Cleveland, Ohio, U.S.A.) using lysis buffer, which consisted of 9M urea (FisherBiotech #BP169-500, Fair Lawn, New Jersey, U.S.A.), 2% Triton-X-100 (iso-octylphenoxypolyethoxyethanol, Pharmacia LKB Biotechnology AB, Cat.No.80-1128-76, Uppsala, Sweden), 2% β-mercaptoethanol (Sigma Cat.No.M-7522, St.Louis, MO) 2% Pharmalyte 3-10 (Pharmacia LKB Biotechnology AB, Cat.No.17-0456-0) and 8mM PMSF (phenylmethylsulfonyl fluoride, Sigma, Oakville, ON, Cat.No.P-7626). The suspension was ultracentrifuged at 200,000 g for 2 hours at 20°C. The protein content of the supernatant was determined using the BioRad protein reagent (BioRad, Mississauga, ON, Cat.No.500-0006) using an adaptation of the Bradford Protein Assay [111]. BSA (bovine serum albumin, Sigma A-7888) standard dilutions were made in a range of 0-24 µg protein in 1 ml volumes of 100 µg/ml stock BSA and 200 µl BioRad protein reagent.

The supernatant containing WG proteins was diluted 1:300 with water and assayed using BioRad protein reagent. After 20 min incubation of the proteins with the BioRad protein reagent, the absorbance of the chromogenic products was read at 595 nm using a microplate reader (BioRad, model 3550-UV). The absorbance values were corrected by subtracting the absorbance reading detected for the lysis buffer alone at a dilution of 1:300. The protein concentration of the WG extract was determined using a standard curve with BSA concentrations ranging from 2-24 μg/ml. The WG extracts were kept frozen at – 20°C in 1 ml aliquots.

1D Western immunoblot

The WG protein extract was run in a Mini-Protein Dual Cell BioRad apparatus. The separation gel was prepared by combining 5 ml of solution C (20 g of acrylamide 99.9% (BioRad Cat.No.161-0101), 0.26 g bis-acrylamide (N,N'-methylene-bis-acrylamide, BioRad Cat No.161-0201) in 100 ml distilled water) plus 2.5 ml of solution A (48 ml, 1N HCl (Baker Analyzed), 36.6 g Trisbase (Sigma 7-9, T1378), 0.23 ml TEMED (N,N, N', N'-tetramethylethylenediamine, BioRad Cat No.161-0801) in 100 ml distilled water] and 2.3 ml distilled water. Once the solution was degassed for 10 min, 100 μl of 10% SDS (sodium dodecyl sulphate, Sigma L-3771) were added, followed by 100 μl of 10% ammonium persulfate (BioRadCat No.161-0700). The stacking gel was composed of 1.25 ml of solution C and 0.95 ml of solution B (48 ml 1N HCl, 5.98 g Trisbase (Sigma 7-9, T1378), 0.46 ml TEMED in 100 ml distilled water) plus 5 ml of distilled water. After degassing, 100 μl of 10% SDS and 100 μl of 10% ammonium persulfate were added. 100 μg of wheat gluten proteins were mixed with sample loading buffer (1000 μl of 10% SDS, 500 μl of 1M Tris-HCL (Sigma T-3253), 200 μl of 100 mM

PMSF (phenylmethylsulfonyl fluoride, Sigma, Cat. No.P-7626), 100 µl of 100 mM EDTA (ethylenediamine tetra acetic acid, Sigma, Cat.No.ED2SS), 1.26 g glycerol (Sigma, Cat.No.G-8773), 1700 µl distilled water, 500 µl mercaptoethanol (Sigma Cat.No.M-7154). WG proteins in sample buffer were denatured by heating for 5 min in a 100°C water bath. Denatured WG proteins were applied to a preparative well along side of a molecular mass marker (10-kDa Ladder, GibcoBRL Cat.No.10064-012). Proteins were resolved by electrophoresis on a 10% SDS-polyacrylamide gel at 180 V for 1-hour [112]. The proteins were electroblotted from the gel onto pure nitrocellulose membranes (pore size 0.45 µm, BioRad, Cat.No.162-0115) [113] at 100 Volts for 1 hour at 4°C. Non-specific binding sites on the membrane were blocked with 5% (w/v) skim milk powder in Tris buffered saline (SMP-TBS), pH 7.5, for one hour. Blots were incubated in sera diluted with SMP-TBS: 1:600 dilution for the BB rat serum cross-sectional study, 1:300 dilution for the BB rat serum prospective study, and 1:50 for human serum. Following 5 x 5 min washes with Tris buffered saline (TBS) containing 1% (v/v) $Tween_{20}$ (Sigma, Cat.No.P2287) the membrane was exposed to peroxidase-conjugated rabbit anti-rat (Dako Diagnostics Canada, Mississauga, ON, Cat.No.P0450) or rabbit anti-human IgG antibody (DAKO, Cat.No.P0214), diluted 1:2000 with SMP-TBS. Membranes were washed 5 x 5 min, incubated with chemiluminescence substrate (ECL-Western blotting detection Amersham Pharmacia Biotech, Buckinghamshire, England, Cat.No.RPN2106) for 1 minute and exposed to Hyperfilm ECL (Amersham Pharmacia Biotech, Cat.No.RPN3114K) for 30 minutes. Hyperfilm ECL was incubated in GBX developer/replenisher (Sigma, Cat.No.P7042) for 30 sec., rinsed in tap water for 5 seconds and incubated in GBX fixer/replenisher (Sigma, Cat.No.P7167) for 30 seconds.

All blots were prepared under the same conditions, with the same lots of blotting membrane, secondary antibody and buffers. Control sera were included on each blot to facilitate comparison among samples. The inter-assay coefficient of variation was 11% and the intra-assay coefficient of variation was 2%.

Total protein stain of WG proteins

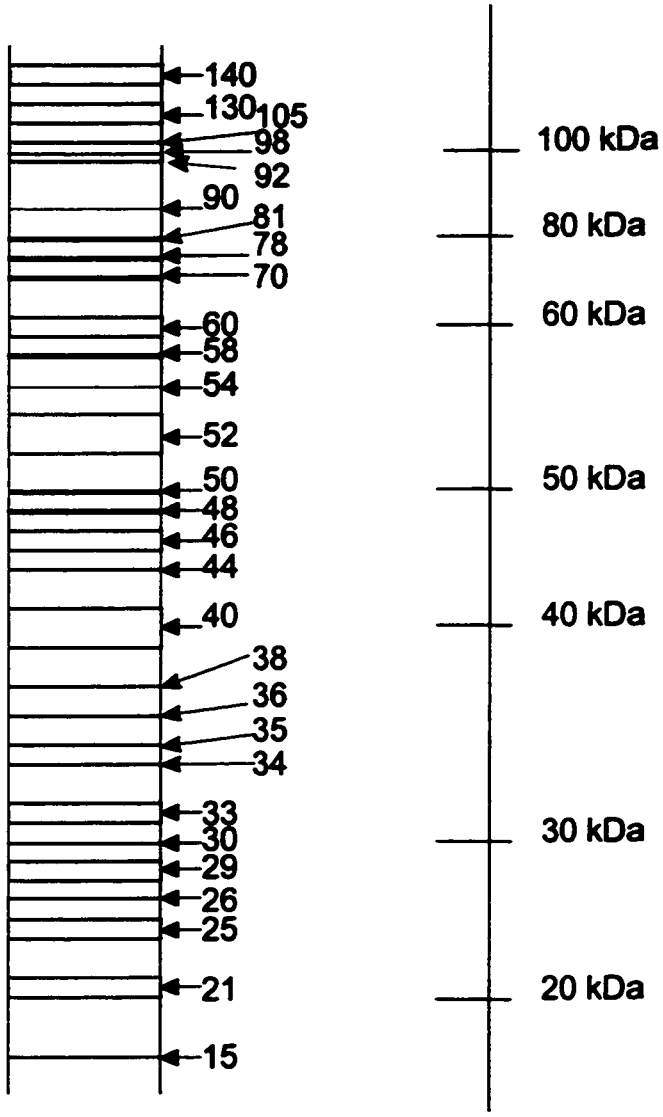
Wheat gluten proteins were resolved by 10% SDS-PAGE as described above (1D Western immunoblot). The gels were stained with silver [114] and the protein bands detected are shown in Figure 1. The antigenic WG protein bands were matched to the molecular mass on the silver stained gel.

Image analysis and statistics of 1D Western blots

Digital images of the Western blot films were acquired using the Kodak Digital Science image station 440CF (Rochester, NY, USA) and analysed using the Kodak Digital Science 1D software (New Haven, CT, USA). The software detected the lanes and bands on each blot using the same detection parameters. The background was sampled in each lane and the average background absorbance was subtracted from the absorbance value for each protein band. The averages of all the absorbance values for each wheat gluten protein band were tabulated and compared between sample populations using one-way ANOVA and LSD post hoc test (STATISTICA Version 4.5, StatSoft Inc., 1993, Tulsa, OK). Fisher's Exact test (2 tailed) was used to compare the frequency of individuals with reactivity to a wheat gluten protein band. The antibody reactivity detected in some serum samples was so high that it prohibited band analysis. These samples were considered outliers and removed from the analysis (Appendix I, page 143).

Figure 1 Summary schematic of wheat gluten protein bands detected by silver stain.

A 9M urea extract of wheat gluten proteins was diluted serially from 5.0 to 0.5 μg and separated on a 10% SDS-PAGE. The gel was stained with a silver stain to visualize the major protein bands. A composite of all the protein bands detected on the silver stained gel is shown as a pictoral illustration. The molecular mass of each wheat proteins band was calculated using Kodak Digital Science 1D image analysis software using a standard molecular mass marker.


140
130
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36
35
34
33
30
29
26
25
21
15
100 kDa
80 kDa
60 kDa
50 kDa
40 kDa
30 kDa
20 kDa

Western blots showed IgG binding to several wheat gluten bands. To assess whether the reactivity to a combination of proteins was related to diabetes, we took into account the frequency of detecting a set of antigenic proteins. The presence of an immunogenic wheat gluten protein band was assigned a value of 1 and the absence of this band was given a value of 0. The sum for a particular band pattern for each animal or patient was tabulated and the frequency of the band pattern was compared among the different populations using Fisher's Exact test (2 tailed).

The specificity and sensitivity of Western blots of wheat gluten proteins were evaluated using the Receiver Operation Characteristic (ROC) plot [115]. Cutoff absorbance values were assigned based on the distribution of absorbances detected in children with diabetes and non-diabetic controls for each protein band. The proportion of patients with absorbances less than the cutoff in HLA matched control sample and the proportion of patients with absorbances greater than the cutoff in the diabetic sample were tabulated and used as estimates of specificity and sensitivity, respectively. The ROC plot was constructed by plotting the sensitivity against the specificity for various cutoff values. A straight-line from (1,0) to (0,1) was drawn on the graph to represent data points that are non-informative diagnostic test markers. The distance from the curve to the individual data points is a measure of the discriminating ability of a particular cutoff value. Different bands with various cutoff values were compared to find the data points that were at the greatest distance from the curve (high sensitivity and high specificity), and thus having the best discriminating ability. In addition, band patterns were evaluated by the "believe-the-positive" rules (positive diagnosis whenever both bands are present and one band exceeds the respective critical OD level) [115].

2D gel electrophoresis of WG

Wheat gluten proteins were solubilized in 9M-urea lysis buffer, as described in the section "wheat gluten extracts for immunoblotting". 150 μg of WG proteins in lysis buffer were added to the IEF rehydration buffer: 4% CHAPS (BioRad, Cat.No.161-0460), 7M urea (BioRad, Cat.No.161-0731), 2M thiourea (Sigma, Cat.No.T7875), 40 mM Trizma base (BioRad, Cat.No.T1503), 2mM tributylphosphine (BioRad163-2101) and 0.4% Bio-lyte 3/10 (BioRad, Cat.No.163-1113). WG in rehydration buffer was centrifuged for 5 min at 21,000 g (L-8M centrifuge, Beckman Instruments Inc. Palo Alto, CA) and 300 μl were pipetted onto each rehydration well. Ready Strips with an immobilised pH gradient (IPG) from pH 3-10 (BioRad, Cat. No. 163-2007) with a linear gradient from pH 3-10 were laid on top of the WG-rehydration buffer for overnight (14-16h) passive rehydration. To prevent evaporation of the solution, the strips were overlaid with mineral oil (Sigma, Cat.No.M5904). The rehydrated strips were placed in the IEF tray with moist filter wicks between the strip and electrodes wire and overlaid with mineral oil. WG proteins were focussed at 21°C at a maximum 80 μAmps for a total of 100,000 V/h on the Protean IEF cell using a four-step ramp of the voltage; 15 min, 250V; 4 h, 5000V; 85000volt/hours; 2h 5000V. After focussing, strips were reduced with 20 mg/ml of DTT (Sigma, Cat.No.D0632) and alkylated with 25 mg/ml iodoacetamide (Sigma, Cat.No.I6125). Wheat proteins were separated in the second dimension according to molecular mass by 10% SDS-PAGE at 30 mAmps for 15 min and 60 mAmps for 3 h using Tris-base/glycine buffer [113].

In 2D-Western blotting, the 2D gels were transferred to pure nitrocellulose membrane (BioRad) in a semi-dry blotter (S&S Pronto, Schleicher&Schuell, Keene, New Hampshire, Cat.No.482121) for 1 hour at 400 mAmps using transfer buffer: 25 mM

Trizma base (Sigma), 192 mM glycine (Fisher Biotech, Fair Lawn, New Jersey, Cat.No.BP381-5) and 20% methanol (Sigma, Cat.No.M1775) [113]. The membranes were washed twice with water and stained with Ponceau S stain to confirm the transfer of proteins. Non-specific binding sites were blocked by adding 5 % (w/v) skim milk powder in TBS with 1% $Tween_{20}$ (Sigma) pH 7.5 to the blots with rocking overnight at 4°C. The membranes were probed with BBdp rat serum at a dilution of 1:500 in blocking buffer for 1 hour at 4°C on a rocker. The membranes were washed on a rocker for 5 x 5 min with TBS containing 1% $Tween_{20}$ (Sigma), final pH, 7.5. The secondary antibody, rabbit anti-rat total IgG labelled with horseradish peroxidase, was diluted to 1:2000 with blocking buffer and incubated with the membranes for 30 min at 4°C on the rocker. The membranes were washed 5 x 5 min with TBS and 1% $Tween_{20}$ (Sigma, pH 7.5) with a final wash of 30 min. The blots were incubated with ECL reagents (Amersham Pharmacia Biotech) and exposed to Hyperfilm ECL (Amersham Pharmacia Biotech) for 90 min. Hyperfilm ECL was incubated in GBX developer/replenisher (Sigma, Cat.No.P7042) for 30 sec., rinsed in tap water for 5 seconds and incubated in GBX fixer/replenisher (Sigma, Cat.No.P7167) for 30 seconds The film images were imported into the Kodak image station (Mandel, Guelph, ON) and analysed using 2D analysis software (PDQuest, BioRad).

Standardisation and controls for Western blotting

The WG protein extract was prepared in a large quantity, followed by a protein assay to reduce variation in the extraction procedure and to ensure consistent amounts of proteins were analysed in each experiment. Aliquots of the WG extract were stored at minus 20°C in 1-ml or 0.2-ml aliquots so that proteins were thawed only once to prevent

protein degradation from repeated freeze-thaw cycles. The ECL-reagents, the secondary antibody and the film developing reagents were tested routinely at each assay to verify their quality. One microlitre of secondary antibody was spotted on a dry nitrocellulose membrane and incubated with ECL-reagents for 1 min. The membrane was wrapped in saran wrap and exposed to film for 10 seconds. The film was developed. These reagents were deemed of good quality when a dark spot was detected on a piece of film at the site where the secondary antibody was spotted on the membrane. The efficiency of the protein transfer to the nitrocellulose membrane was evaluated by staining the membrane with 1% (w/v) Ponceau S stain (Sigma, Cat.No.P3504) and staining the gel post-transfer with 1% Coomassie blue in methanol. The Coomassie blue stain routinely showed that little protein remained in the gel, while proteins on the membrane were always detected using the Ponceau S stain. Non-specific binding of secondary antibody to the proteins was ruled out by omitting the primary antibody (serum) from the Western blot method. Non-specific binding of primary antibody to WG proteins was ruled out by using corn as an antigen.

To reduce the effects of blot to blot variation in our semi-quantitative analysis, several inter- and intra-assay controls were used: 1) the background on each blot was subtracted from the signal detected; 2) the band detection parameters such as sensitivity, minimum density, noise filter, shoulder sensitivity and size scale to standard values were all customised to suite the range of values in all images and thence kept constant for all analyses; 3) there were two control lanes on each blot in each experiment that were probed with the same pooled BBc serum sample (pooled from 5 control BBc rats); 4) whenever possible, entire experimental groups were tested in one day to maintain the

same conditions across the group; 5) large volumes of solutions were prepared in 10X stock concentrates to reduce slight changes in running conditions.

Mass spectrometry analysis

For proteome analysis, a series of 2D gels of WG proteins (38, 75, 150 and 300 μg) were prepared and stained with a non-fixing silver stain [116]. Proteins of interest were excised and analysed by mass spectrometry at the National Research Council of Canada.

In-gel tryptic digestion was carried out using the ProGest™ automatic digestor (Genomic Solutions, Ann Arbor, MI). The silver stained protein gel spots were loaded into a 96-well polypropylene plate specially designed to fit the ProGest™. The digestor program carried out the following steps. The gel pieces were destained with 1:1 ratio of 30 mM potassium ferricyanide (Sigma) and 100 mM sodium thiosulfate (Sigma) according to the method of Gharahdaghi *et al.* [116]. Destaining was usually complete within 5 minutes after which the gel spots were rinsed with deionized water. The gel spots were then shrunk with acetonitrile (Optimal Grade, Fisher Scientific, Nepean, ON), reswollen with digestion buffer (20 μL of 50 mM ammonium bicarbonate (BHD, Mississauga, ON) containing 200 ng of modified sequencing grade trypsin (Promega Sequencing Grade Modified Trypsin, Promega, Madison, WI, Cat.No.V5113). Sufficient 50 mM ammonium bicarbonate (BDH) was added to each tube to cover the rehydrated gel pieces (50 μL). The Eppendorf tubes were then sealed and digestion was carried out overnight at 37^0C.

After digestion, the tryptic peptides were recovered as follows. The buffer solution surrounding the gel pieces was transferred to another 96-well plate. The gel

pieces were extracted first with 50 μL of 5% acetic acid (Anachemica, Mississauga, ON) and then with 50 μL of 5% acetic acid (Anachemica) in 50% methanol (OmniSolv, Merck, Germany). The extracts and the original digest buffer were pooled, evaporated to dryness on a centrifugal evaporator (Savant) and stored at -20^0C until required for analysis.

Capillary LC-MS/MS (capLC-MS/MS) was carried out using a Waters CapLC liquid chromatograph (Waters, Milford, MA, U.S.A.) coupled to a Q-TOF 2 mass spectrometer (Micromass, Manchester, UK) using an electrospray ionization interface. The digest extracts were redissolved in 15 μL of 5% (v/v) acetonitrile (Fischer Scientific), 0.5% acetic acid (Anachemica) and 10 μL was loaded for each analysis. The tryptic peptides were concentrated on a 0.3 mm i.d. x 5 mm μ-precolumn cartridge (LC-Packings, San Francisco, CA, U.S.A.). Rapid peptide elution was achieved using a linear gradient of 5 to 60% acetonitrile (Fischer Scientific), 0.2% formic acid in 6 minutes (flow rate of 1 μL/min).

The mass spectrometer was operated in data dependent acquisition mode. Briefly, a fast survey scan is carried out to profile the ions entering the instrument. The instrument was programmed to switch to MS/MS sequencing mode upon detection of doubly and triply protonated ions (a background of singly charged ions is constantly observed and arises form the HPLC solvent). Three multiply-charged ions could be selected per survey scan and MS/MS scans were acquired for 2 seconds on each ion selected. The collision gas was nitrogen and the collision offset (i.e., the voltage gradient across the collision cell) was automatically selected. The instrument returns to survey scan mode after the MS/MS spectra have been acquired.

Database Searching

The peptide MS/MS spectra acquired for each protein sample were converted to peal list files (.PKL files) using the instrument manufacturer's proprietary software (ProteinLynx). Database searching was carried out automatically using Mascot™ (Matrix Science, London, U.K.). This program attempts to identify the protein by using the peak list files to search protein and nucleotide databases for matching sequences. In this instance all searches were carried out against the NCBI protein sequence database [79].

In some cases the spectra could not be matched and the sequencing had to be done manually. The information acquired in this manner was used to search the NCBI's protein sequence database using MS-Seq (Protein Prospector™, Mass Spectrometry Facility, UCSF, San Francisco, CA, U.S.A.) or NCBI's BLAST searching algorithms.

RESULTS

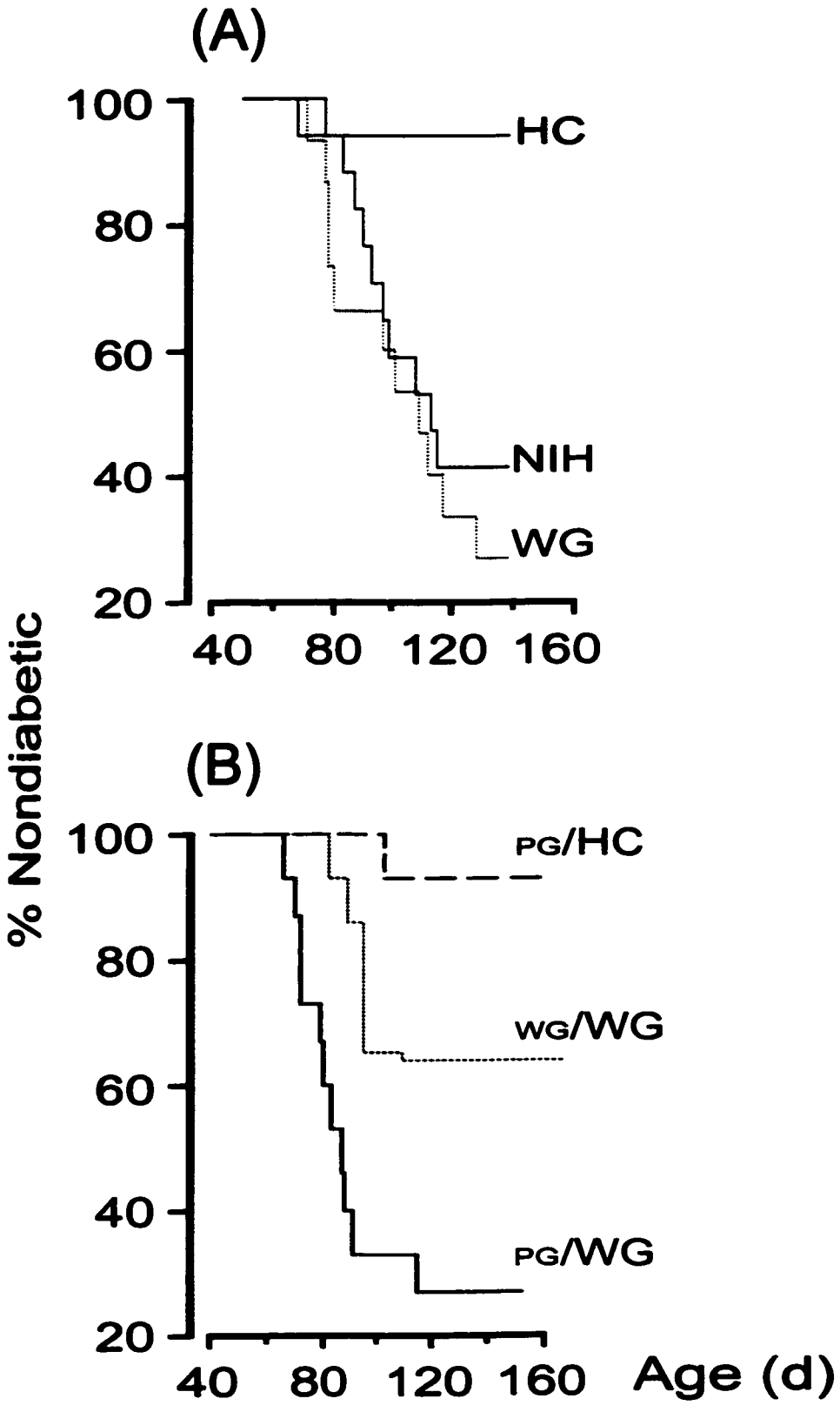
Study # 1: Induction of early oral tolerance to WG proteins

Dietary antigens, particularly from cereal based diets such as the open-formula standard rodent diet, NIH-07, have been reported to induce type 1 diabetes (T1D) when fed to BBdp rats and NOD mice [40]. Groups of 15-17 BBdp animals were weaned at day 23 onto three diet formulations, hydrolysed casein (HC, diabetes retardant diet, negative control), NIH-07 (cereal-based, diabetes-promoting diet, positive control) and WG diet. Both HC and the WG semipurified diets were nutritionally complete, isocaloric, isonitrogenous balanced diet formulations supplemented with 0.02% DL-methionine, 0.5% lysine, and 0.08% L-threonine, the major difference being the source of amino acids [40]. The survival curves depicted in Figure 2, Panel A, show that feeding cereal based diets, WG and NIH-07 resulted in more diabetes cases compared with the HC diet. The frequency of diabetes cases in BBdp rats fed the WG or NIH diets was 73% or 59% respectively at 140 days. Only 6% of HC-fed animals developed diabetes by 140 d. The results are consistent with previous studies showing that changing the amino acid source in these susceptible animals modified the development of T1D. WG proteins in the diet had a high diabetes inducing potential when fed to genetically susceptible rodents. These data imply that components of wheat gluten can promote development of T1D. We postulate that certain WG proteins in the diet provide information that results in an inappropriate β-cell specific immune response in susceptible individuals.

Figure 2 Survival graphs of BBdp rats fed NIH, HC and WG diets.

Panel A: BBdp rats were weaned onto NIH, WG or HC diet. WG and NIH induced the highest incidence of diabetes. HC fed animals had the lowest incidence of diabetes. Each group consisted of 15-17 animals. Animals diagnosed with T1D were killed and the results are reported as a standard survival graph commonly used to analyze treatment effects in animals that develop spontaneous T1D.

Panel B: Early oral exposure of BBdp rats from day 4 to 7 to WG in Pregestimil or Pregestimil alone, denoted in small font. The weaning diet is shown in standard upper case font. The animal numbers per group were as follows: PG/WG, 14; WG/WG, 15; and PG/HC, 15. Early exposure to WG, delayed onset and inhibited development of diabetes in 50% of rats. WG/WG versus PG/WG, p=0.008 (Kaplan-Meier, log rank statistics) (Scott et. al. Jan. 2002, reference no. 171).


(A)
100
80
60
40
20
HC
NIH
WG
40
80
120
160
(B)
% Nondiabetic
100
80
60
40
20
PG/HC
WG/WG
PG/WG
40
80
120
160
Age (d)

The gut of diabetes prone rodents may respond differently to WG proteins compared with HC peptides, influencing immune regulation, beta cell mass and function, and the development of diabetes. Hence, if we educate the gut immune system of the neonate, which is less well developed than in adults, to tolerate WG proteins, then we might inhibit the development of T1D.

Our aim in the following feeding study was to modify WG-related immune reactivity in the gut by early oral feeding of BBdp rat pups with wheat proteins. The rat pups were removed from the dams twice daily for a short time on day 4 to 7 (day of birth was counted as day 1) and fed orally with WG proteins in Pregestimil® (PG) or with PG alone. PG is a hydrolysed casein-based (hypoallergenic) infant formula. Early oral feeding of WG in Pregestimil® delayed the onset of diabetes and showed a marked improvement in survival compared with animals fed vehicle alone and weaned onto the wheat gluten diet (Figure 2 panel B). 34% of WG-fed neonates developed diabetes, whereas 67% of those dosed with vehicle alone became diabetic ($p=0.05$). In the case of animals fed PG in the first week of life and weaned onto the HC diet, only 7% developed diabetes. These findings suggest that exposure to diabetes-promoting WG proteins during infancy protected a proportion (~1/2) of animals from developing diabetes as adults. We also noted that onset of diabetes was delayed (82 vs 91 days) in animals that received early oral WG proteins. The data support our hypothesis that the gut immune system has the potential to modify the progression of T1D. The benefit of neonatal treatment may be the result of changes in the local immune responses in the gastrointestinal tract.

Study #2: Gut cytokines

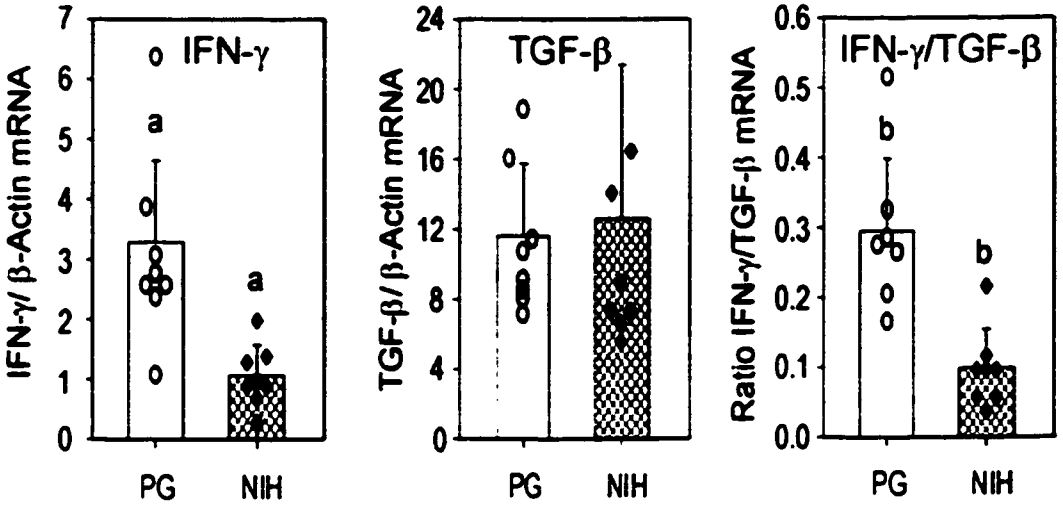
To examine whether neonatal exposure to dietary antigens of different diabetes-promoting potential are associated with changes in immune response, we measured cytokine profiles in the duodenal section of the small intestine using RT-PCR. We measured IFN-γ and TGF-β gene expression in the gut of 5 day old BBdp rat pups fed PG or PG+NIH (n=8/group) and euthanized 24 hours later. The intestine of rat pups fed PG+NIH did not show changes in TGF-β mRNA expression. We detected a down regulation in IFN-γ in PG+NIH-fed animals compared to those fed PG alone and the IFN-γ/TGF-β ratio was decreased. This finding reflected changes in local gut immune bias 24 hours after oral exposure to PG+NIH in neonatal BBdp rats (Figure 3). The relationship between the gut immune system, wheat proteins and the pancreas may become clearer after the molecular characterisation of the WG proteins that are implicated in the pathogenesis of T1D.

Study #3: 1D WG Western blots with BB rat serum

Our data from Study #1 suggest that WG is a potent modulator of the development of T1D. Animals that were fed a WG diet from weaning developed a high incidence of diabetes, whereas fewer animals developed T1D when they were fed with these proteins during infancy. The molecular identity of these components has not been examined and needs to be clarified.

Figure 3 Gut cytokines following neonatal exposure of BBdp rats to NIH diet

Five day old BBdp rat pups were fed Pregestimil (PG) or PG+NIH (NIH) diet and euthanized 24 hours later. Tissue from the upper small intestine was removed, snap frozen and RNA was isolated for semi-quantitative RT-PCR analysis. n=8/group ANOVA, values sharing letters a, $p=0.00002$; b, $p=0.0003$. Values are expressed as mean $\pm$ SD. (Scott et.al. Jan. 2002, reference no. 171).


IFN-γ
IFN-γ/ β-Actin mRNA
7
6
5
4
3
2
1
0
a
a
PG
NIH
TGF-β
TGF-β/ β-Actin mRNA
24
20
16
12
8
4
0
PG
NIH
IFN-γ/TGF-β
Ratio IFN-γ/TGF-β mRNA
0.6
0.5
0.4
0.3
0.2
0.1
0.0
b
b
PG
NIH

There are several compelling reasons to characterise diabetes related WG proteins: 1) systemic antibody response to certain WG proteins may be different in progressors (pre-diabetes) and non-progressors (asymptomatic), which could reflect abnormal T cell activity in these individuals; 2) antibodies to WG proteins may be a prognostic marker of disease progression in susceptible individuals; 3) anti-pancreas autoimmunity may be gluten dependent because anti-pancreas antibodies disappeared in patients with T1D on a gluten-free diet [77]; and 4) T1D has been linked with celiac disease, which suggests that the aetiology of T1D may resemble in some ways the wheat-induced, immune mediated attack in the gut of CD patients and draws attention to the role of the gut in diabetes pathogenesis [73, 117].

Why study IgG antibody reactivity? Some authors have reported that destructive autoimmunity, which has been associated with Th1-dominated autoimmune responses, arises late in the disease process. They suggest that Th2 islet autoimmunity, which is associated with humoral reactivity to islet antigens, precedes the aggressive Th1 autoimmunity [118, 119]. Other reports suggest that the autoimmune response starts as a pure Th1 immune attack against a primary islet autoantigen, which is then amplified and spreads to other islet antigens as the β-cell destruction progresses [120, 121]. Anti-inflammatory Th2 responses are also induced to the primary islet autoantigen and likewise antibody reactivity spreads to other antigens. Islet autoantibodies of the IgG class are used as serological markers of disease progression and mathematical models have been proposed to estimate the time to diabetes onset [122]. Because, the subclass distribution of IgG antibodies to ICA is heterogeneous [123] and investigation of plasma IgG reactivity to the 64 kDa antigen led to the discovery of GAD, a major autoantigen in

T1D [84, 124], we used a similar strategy to investigate total IgG antibody reactivity to WG proteins.

A commercial WG preparation (ICN) was extracted with 9M urea lysis buffer to obtain a total protein isolate of WG proteins. The protein isolate was separated by molecular mass on a 10% SDS-gel and the protein bands were detected with a silver stain [114]. Each band was assigned a molecular mass based on the molecular mass standard. The WG protein bands that were detected are shown in Figure 1.

The anti-WG antibodies in BB rat serum appear to be specific for WG proteins as demonstrated by: (1) serum antibodies from pre-diabetic and asymptomatic rats (70 day old) that recognise WG proteins do not bind to corn proteins at a dilution of 1:600 (Figure 4) and (2) anti-WG antibodies in serum from a diabetic BBdp rat bound to WG proteins at a dilution as high as 1:2,500 (Figure 5), which suggests that these are high affinity anti-WG antibodies.

We compared the antigenicity of WG proteins in animals at different genetic risk for T1D. We examined the IgG antibody reactivity to WG proteins in sera from diabetes resistant, BBc, and diabetes-prone, BBdp rats. NIH-07 fed rats from two age groups were selected, 50 day old and 70 day old, serum was collected and used to probe the WG protein extracts on Western immunoblots.

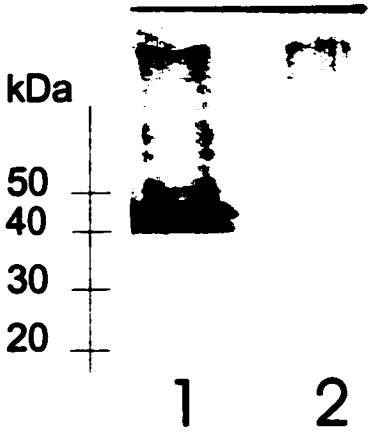
Western blots of WG proteins probed with serum from 50 and 70-day old BBc rats at a dilution of 1:600 did not show any rat IgG antibody binding (Figure 6). In contrast, when WG proteins were probed with serum from 50-day-old BBdp rats, a weak signal (~0.1 OD) was detected for a few WG protein bands.

Figure 4 Western blot of wheat gluten and corn proteins

10 μg of wheat gluten or corn proteins were resolved by 10% SDS-PAGE and blotted onto a pure nitrocellulose membrane. The membrane was incubated with 70 day BBdp rat serum (asymptomatic, upper panel or pre-diabetic, lower panel) at 1:600 dilution in blocking buffer and 1:2000 dilution of anti-rat total IgG antibody labeled with HRP. Antibody binding was detected by ECL. No antibody binding to corn proteins was detected.

Lane 1, wheat gluten proteins (ICN) extracted with 9M urea extraction buffer
Lane 2, corn proteins extracted with 9M urea extraction buffer

Asymptomatic BBdp serum (70d)



Pre-diabetic BBdp serum (70d)

Figure 5 Serial dilution of serum anti-WG antibodies

Western blot of wheat gluten proteins probed with serially diluted serum from a diabetic BBdp rat. IgG binding detected by enhanced chemiluminescence.

Serial dilution of BB rat serum diluted with blocking buffer:
Lane 1, 1: 10
Lane 2, 1: 40
Lane 3, 1: 160
Lane 4, 1: 640
Lane 5, 1: 2500

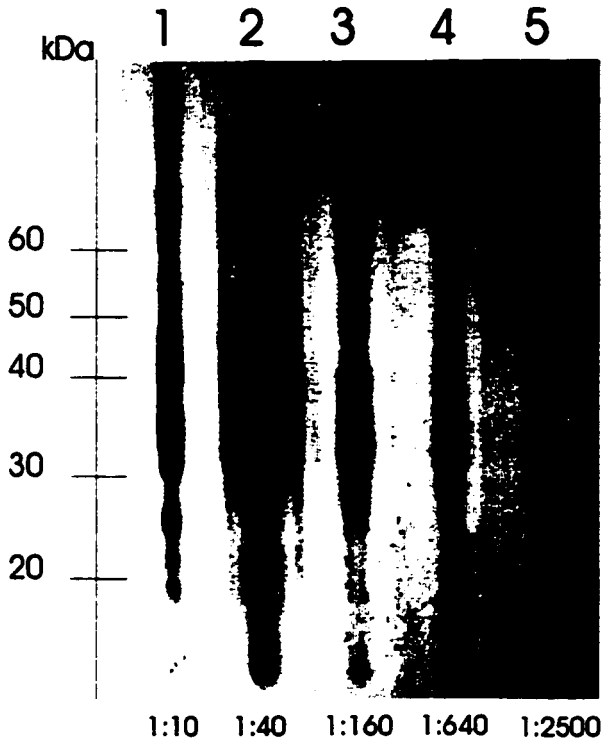
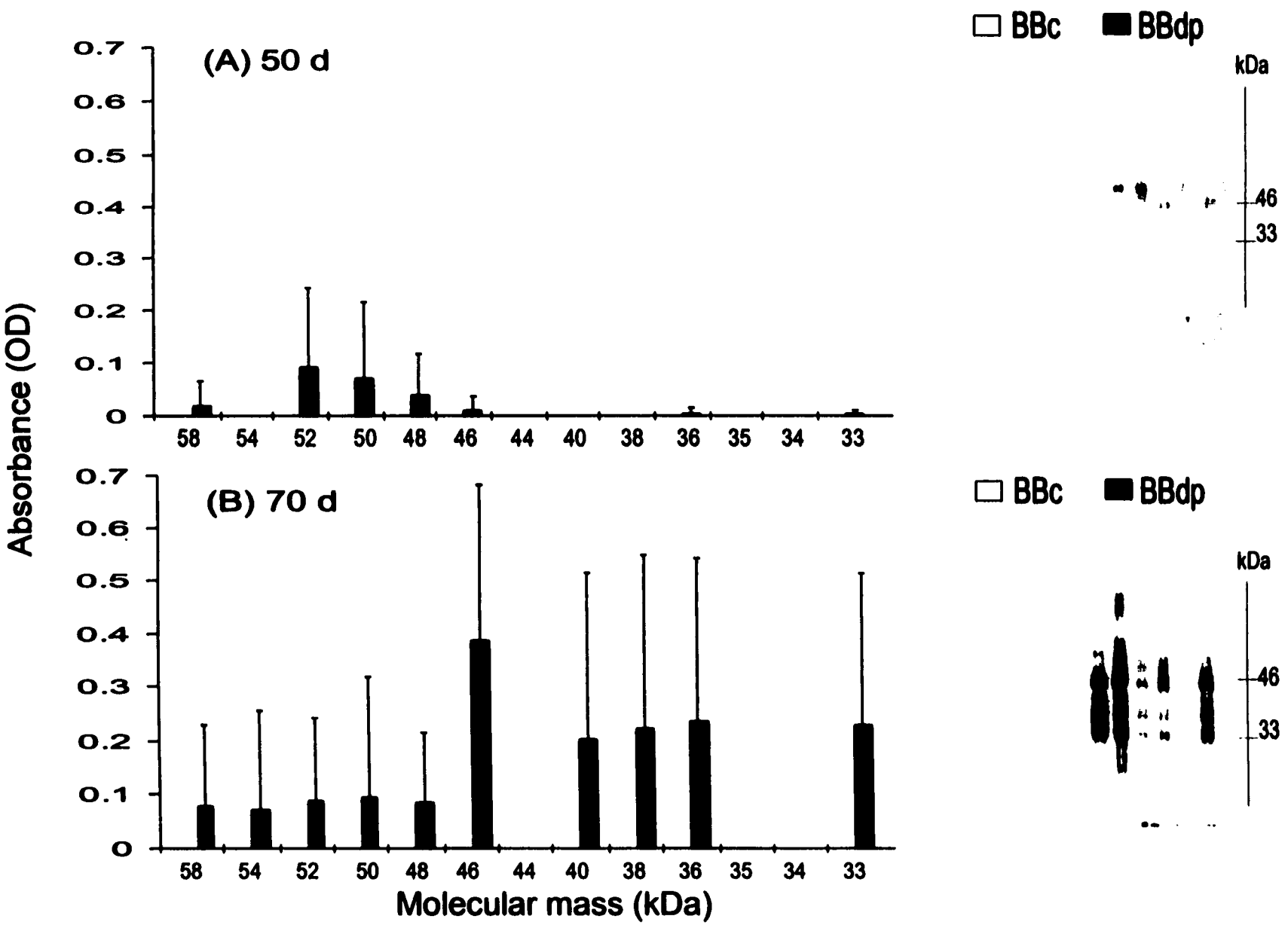

kDa
1
2
3
4
5
60
50
40
30
20
1:10
1:40
1:160
1:640
1:2500

Figure 6 Western blot of wheat gluten proteins probed with cross-sectional serum samples from NIH-fed BBdp and BBc rats.

BBdp (n=12) and BBc (n=10) rats fed an NIH diet were euthanized at 50 days of age (Panel A) or 70 days (Panel B) and a 1:600 dilution of the individual serum samples was used to probe a Western blot of wheat gluten proteins extracted using 9M urea lysis buffer. IgG antibody binding was detected using an anti-rat total IgG antibody labeled with HRP, which was visualized using enhanced chemiluminescence. Mean absorbance of each wheat gluten protein band is shown in bar graphs and representative Western blots are shown to the right. Values are expressed as mean $\pm$ SD.

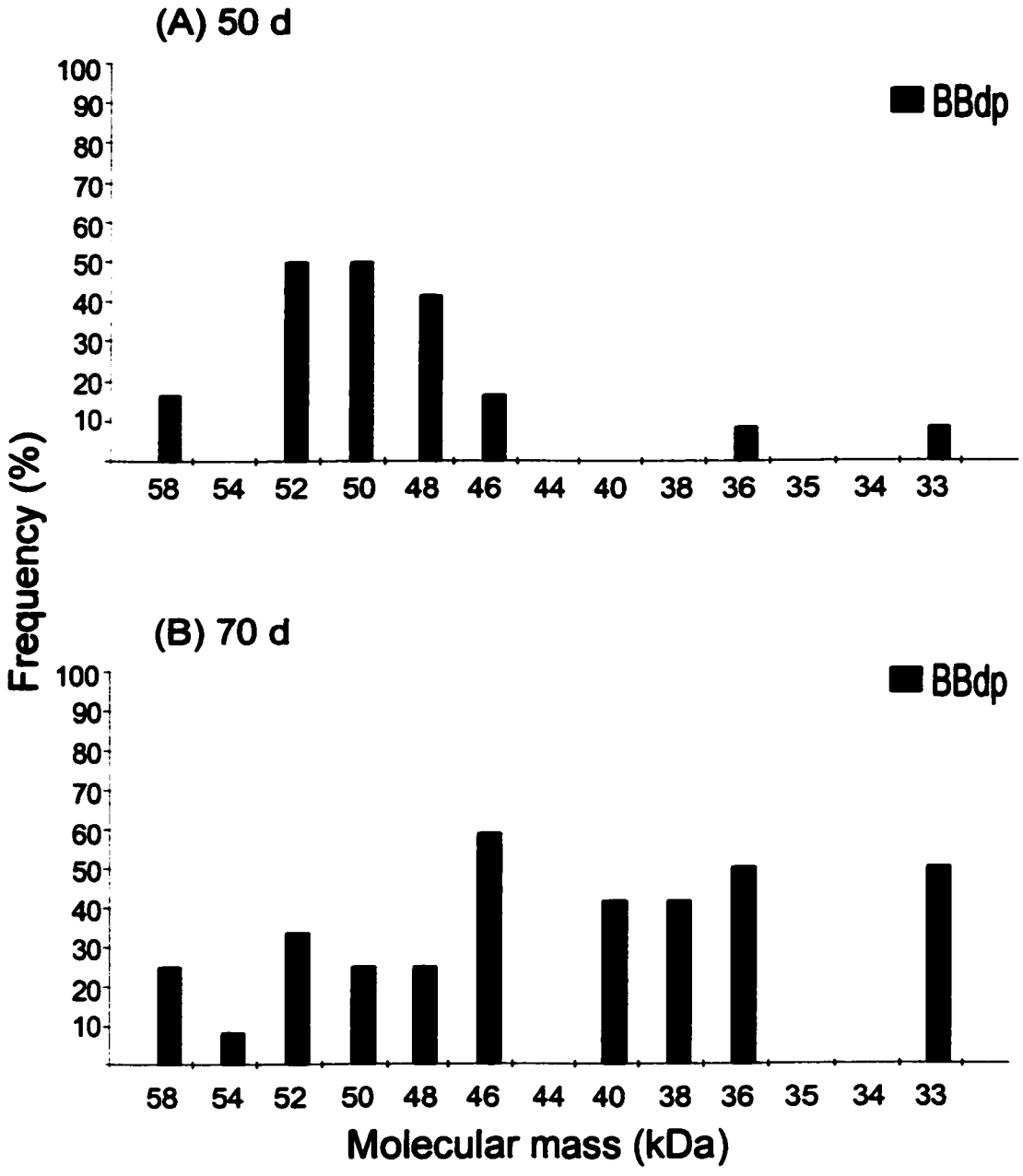

(A) 50 d
(B) 70 d
Absorbance (OD)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
58 54 52 50 48 46 44 40 38 36 35 34 33
Molecular mass (kDa)
BBc
BBdp
kDa
46
33

In particular, 50% of BBdp rats (6/12) reacted weakly (0.07-0.09 OD) to two WG protein bands, 50 and 52 kDa (Figure 7, Panel A). At 70 days, BBdp rats reacted to several more WG bands: 33, 36, 38, 40 and 46 kDa proteins (0.2-0.38 OD) (Figure 6, Panel B). Despite the higher absorbance of these bands, the frequency of BBdp rats reacting to any one of them was 40-50%, with the exception of the 46 kDa band, which was detected in 58% of BBdp rats (Figure 7 Panel B). A weak signal was also detected for 48, 50, 52, 54, 58 and 70 kDa proteins (<0.1OD) but the frequency of animals responding to these proteins was 10-35%.

We considered whether reactivity to multiple bands was associated with an increased risk of diabetes (Table 1). The frequency of antibody reactivity to WG proteins was similar in 50 d BBc and BBdp rats. At 70 days, a higher frequency of antibody binding to a series of low molecular mass proteins (33, 36, 38, 40 and 46 kDa) was associated with increased risk of diabetes. This band pattern was detected in 58% (7/12) of 70 d BBdp rats but was not observed in 70 d BBc rats (0/10, p=0.0046). Two wheat protein bands, 33+36 kDa were most frequently detected in 70 d BBdp rats (8/12) but were undetectable in Western blots probed with 70d BBc serum (0/10, p=0.0015). Taken together, the results of the cross-sectional study suggested that there are differences in the humoral reactivity to wheat gluten proteins in animals at different risk of developing diabetes. Increased antibody binding to WG proteins was associated with higher risk of diabetes (BBdp>BBc). The difference in immunoreactivity may reflect an inherent difference in the processing of antigens in the gut of BBdp animals, and/or differences in the immune regulation in the gut associated lymphoid tissue (GALT) of these animals.

Figure 7 Percentage of 50 day and 70 day old BBdp and BBc rats fed NIH-07 with IgG antibodies to wheat gluten proteins.

BBdp (n=12) and BBc (n=10) rats fed an NIH-07 diet were euthanized at 50 days of age (Panel A) or 70 days (Panel B) and a 1:600 dilution of the individual serum samples was used to probe a Western blot of wheat gluten proteins extracted using 9M urea lysis buffer. IgG antibody binding was detected using an anti-rat total IgG antibody labeled with HRP, which was visualized by enhanced chemiluminescence. Frequency of animals that reacted to individual wheat gluten protein bands is shown in bar graphs.



Note: No bands detected using BBc serum (1:600)

Table 1: Frequency of signal detected against combinations of WG proteins in cross-sectional BBdp and BBc rats fed NIH-07

Bands	Comparison	Age	BBc n=10 n(%)	BBdp n=12 n(%)	Band pattern p-value
38+33	BBc vs BBdp	50d	0(0)	0(0)	1
38+36+33	BBc vs BBdp	50d	0(0)	0(0)	1
36+33	BBc vs BBdp	50d	0(0)	1(8)	0.54
40+38+36+33	BBc vs BBdp	50d	0(0)	0(0)	1
52+50+48	BBc vs BBdp	50d	0(0)	4(33)	0.067
46+40+38+36+33	BBc vs BBdp	50d	0(0)	0(0)	1
38+33	BBc vs BBdp	70d	0(0)	7(58)	0.0046
38+36+33	BBc vs BBdp	70d	0(0)	7(58)	0.0046
36+33	BBc vs BBdp	70d	0(0)	8(67)	0.0015
40+38+36+33	BBc vs BBdp	70d	0(0)	7(58)	0.0046
52+50+48	BBc vs BBdp	70d	0(0)	1(8)	0.54
46+40+38+36+33	BBc vs BBdp	70d	0(0)	7(58)	0.0046

Because the cross-sectional study showed that genetic risk of T1D was associated with increased antibody reactivity to WG, we wanted to examine whether differences in IgG antibody binding to WG proteins were associated with the development of the disease. The limitation of the previous cross-sectional study was that we could not predict which of the diabetes-prone animals would become diabetic. "Pre-diabetic" animals can only be identified retrospectively, after clinical diagnosis of the disease. Therefore, we took prospective serum samples at 50 and 70 days from BBdp rats fed a WG diet and classified them as "pre-diabetic" or "asymptomatic". Serum samples were collected from two feeding studies, referenced as 84F and 86F.

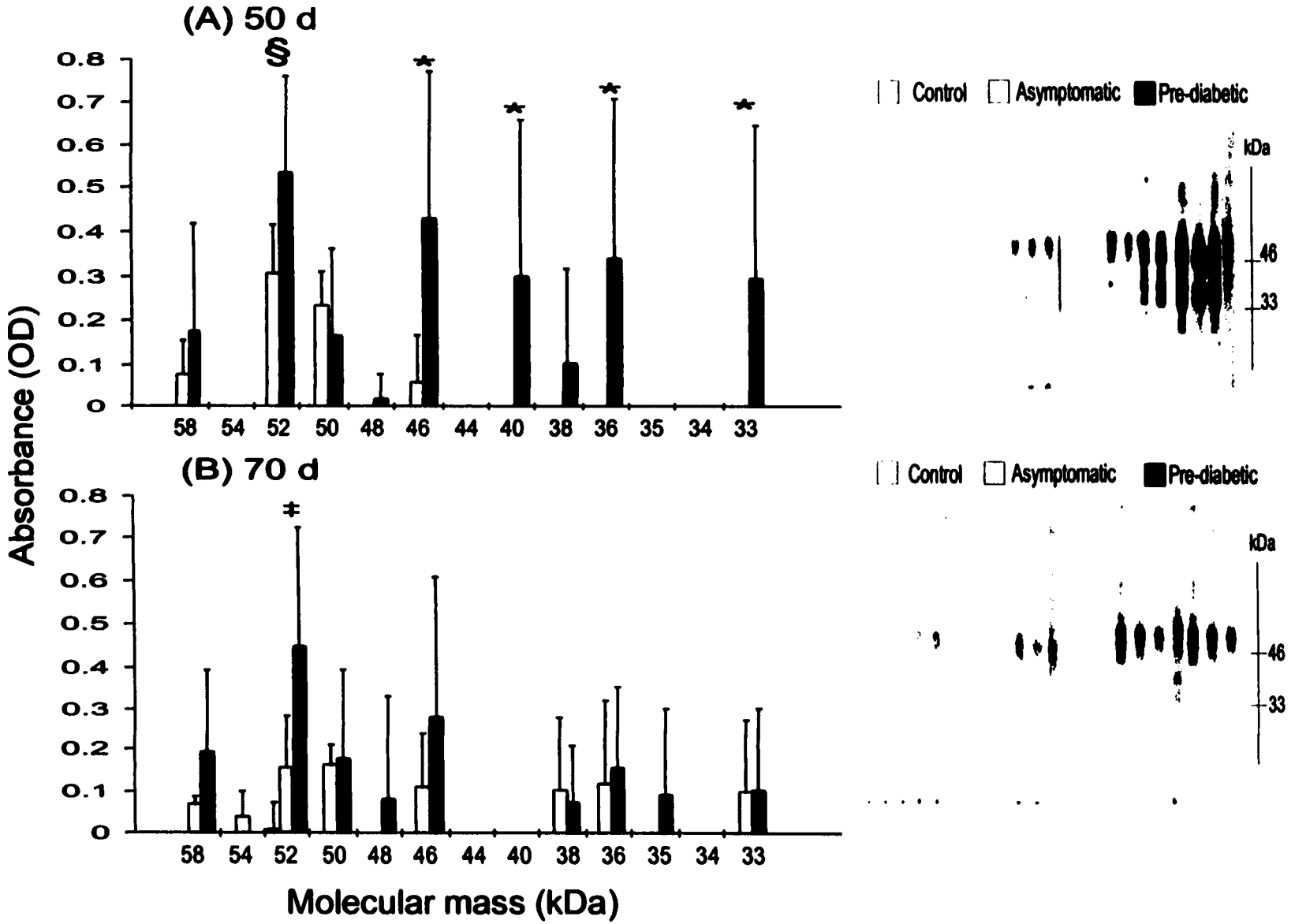
In the 84F study, BBc (n=10) and BBdp rats (n=15) were weaned starting at around day 23 onto a wheat gluten diet. Western blots of WG proteins were probed with anti-serum obtained at 50 and 70 days from 10 BBc, 4 asymptomatic BBdp animals and 11 pre-diabetic BBdp animals. One asymptomatic rat was an outlier because the level of reactivity to all WG proteins was such that we could not resolve individual protein bands in the Western blot analysis (Appendix I, page 143).

The major imunoreactive proteins in 50 day pre-diabetic BBdp rats were 33, 36, 40 and 46 kDa ($p=0.01$-0.03, Figure 8, Panel A). Eighty percent of pre-diabetic animals (9/11) had antibodies to 33, 36, and 46 kDa bands, 55% (6/11) responded to 40 kDa proteins, whereas 33% (1/3) of the asymptomatic animals showed low reactivity ($OD<0.1$) to only one of these bands, the 46 kDa proteins (Figure 9, Panel A).

At 70 days, we could no longer detect differences in the level of reactivity to low molecular mass WG proteins of 33-46 kDa in pre-diabetic and asymptomatic BBdp rats.

Figure 8 Western blot of wheat gluten proteins probed with serum samples taken at 50 and 70 days from BBdp rats fed wheat gluten (84F).

Serum samples from pre-diabetic (n=11), asymptomatic (n=3) BBdp and diabetes-resistant (control) BBc rats (n=10) fed a WG diet were taken at 50 days (Panel A) and 70 days (Panel B) from each animal. Western blots of wheat gluten proteins extracted with 9M urea lysis buffer were probed with individual serum samples. IgG antibody binding was detected using an anti-rat total IgG antibody labeled with HRP, which was visualized by enhanced chemiluminescence. Mean absorbance $\pm$ SD of each wheat gluten protein band is shown in bar graphs. Representative blots are shown on the right. ANOVA post hoc comparisons of means using LSD test of 50d pre-diabetic and asymptomatic BBdp rats; § indicates p=0.05, ✱ indicates p=0.01-0.03. In LSD test of 70 d pre-diabetic and asymptomatic BBdp rats; ‡ indicates p=0.01, ✦ indicates p<0.002.


(A) 50 d
§
*
*
*
*
(B) 70 d
‡
Absorbance (OD)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
58 54 52 50 48 46 44 40 38 36 35 34 33
Molecular mass (kDa)
Control
Asymptomatic
Pre-diabetic
kDa
46
33

The mean signal intensity detected for these proteins was 0.05-0.3 OD (Figure 8 Panel B, $p>0.05$). The trend suggested that 33 and 36 kDa WG proteins were more frequently antigenic in 72% (8/11) pre-diabetic compared with 33% (1/3) of asymptomatic animals (Figure 9 Panel B). None of the 50 or 70 d BBc rats showed IgG antibody binding to these proteins. Together the data suggest that IgG binding to low molecular mass proteins is higher and more frequently detected in 50 d animals.

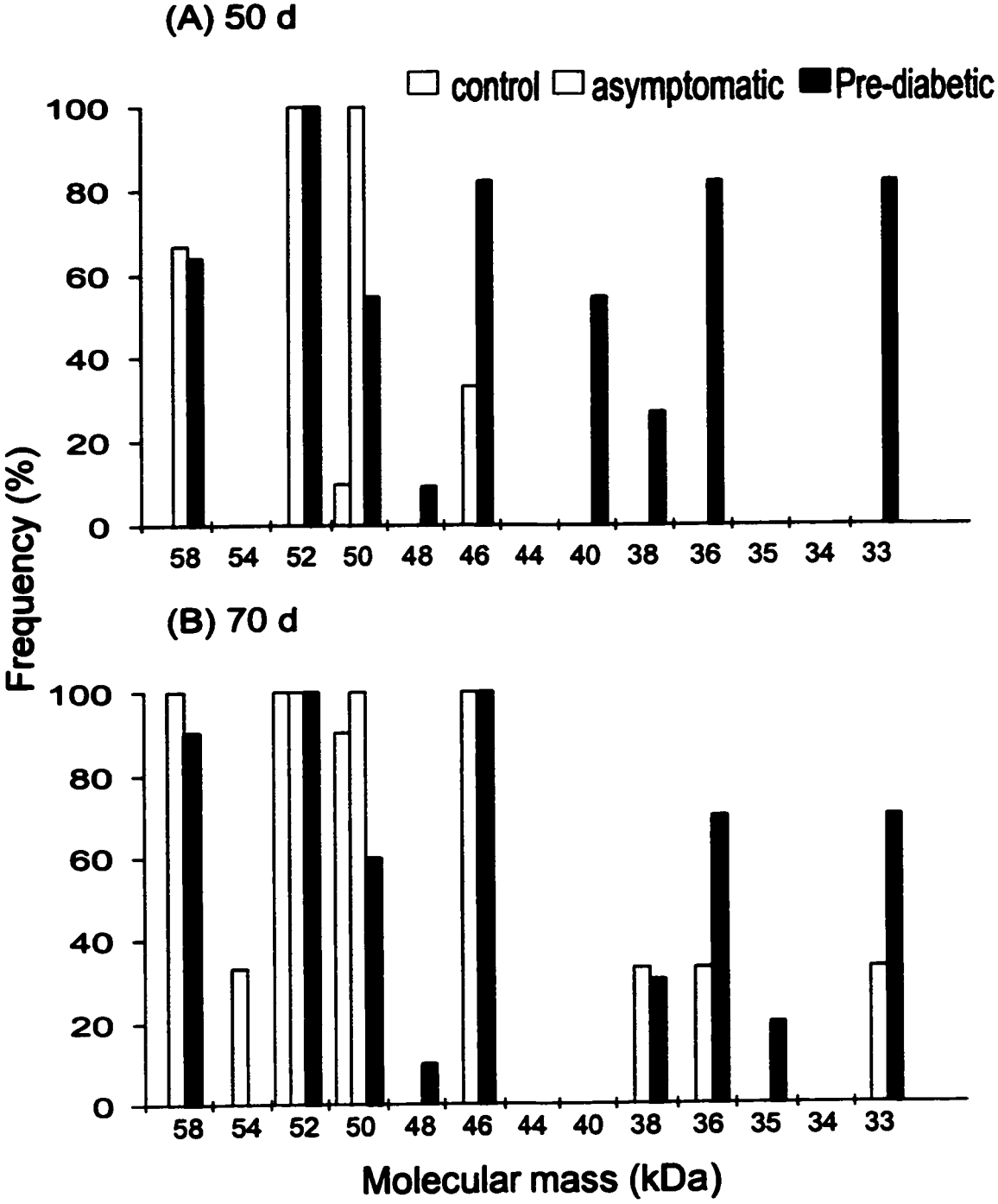
Figure 8 Panel A, shows a trend that 50 d pre-diabetic rats had higher level of antibody binding to 52 kDa proteins compared with asymptomatic BBdp rats ($p=0.05$). There was no difference in the frequency of reactivity in asymptomatic and pre-diabetic BBdp animals (3/3 asymptomatic and 11/11 pre-diabetic rats, Figure 9 Panel A). However, none of the WG-fed BBc rats responded to these proteins (0/10 BBc, Figure 9, Panel A).

At 70 days, we detected a higher level of reactivity to 52 kDa proteins (0.45 OD) in pre-diabetic BBdp rats compared with asymptomatic animals (0.18 OD, $p=0.01$, Figure 8 Panel B). However, the frequency of reactivity in asymptomatic (3/3), pre-diabetic rats (11/11), and BBc (10/10) rats was similar. Taken together, at 70 days, the higher frequency and level of antibody binding to 52 kDa proteins in BBdp compared with BBc rats were associated with increased risk of diabetes.

All asymptomatic 50d BBdp rats (3/3) reacted to 50 kDa WG proteins in comparison with 50% of pre-diabetic rats (6/11) and 10% of BBc rats (1/10, Figure 9 Panel A).

Figure 9 Frequency of 50 and 70 day old BBdp rats with IgG antibodies to wheat gluten proteins (84F).

Serum samples from pre-diabetic (n=11) and asymptomatic (n=3) BBdp and diabetes resistant (control) BBc (n=10) rats fed a WG diet were taken at 50 days (Panel A) and 70 days (Panel B) from each animal. Western blots of wheat gluten proteins extracted with 9M urea lysis buffer were probed with individual serum samples. IgG antibody binding was detected using an anti-rat total IgG antibody labeled with HRP and visualized by enhanced chemiluminescence.


(A) 50 d
control
asymptomatic
Pre-diabetic
100
80
60
40
20
0
58 54 52 50 48 46 44 40 38 36 35 34 33
(B) 70 d
Frequency (%)
Molecular mass (kDa)

However, the level of reactivity was similar in 50 and 70 d asymptomatic and pre-diabetic BBdp rats (~0.2 OD, Figure 8 Panel A). Ninety percent of 70 day old BBc animals (9/10, Figure 9 Panel A) showed weak reactivity to 50 kDa proteins (<0.05 OD, Figure 8 Panel A).

We examined whether concomitant reactivity against several WG proteins was related to diabetes development. Table 2 summarises various combinations of protein bands that were immunoreactive in asymptomatic, pre-diabetic BBdp and BBc rats. IgG antibody binding to 33 and 36 kDa at 50 days tended to be more frequently detected in pre-diabetic BBdp rats (64%, 7/11) compared with asymptomatic animals (0/3, p=0.1). However this was only a trend and not statistically significant due to the small number of asymptomatic animals (n=3). Another trend of note was the combination of immunogenic proteins 46+52 kDa. This combination of wheat proteins was immunogenic in 82% of 50 d pre-diabetic animals (9/11) compared with 33% of asymptomatic (1/3, p=0.2). There were no differences in the frequency of reactivity to this band pattern at 70 d in BBdp rats (11/11 pre-diabetic and 3/3 asymptomatic).

A comparison of BBc and pre-diabetic BBdp rats at 50 d and 70 d showed that the presence of IgG antibodies against 46+52 kDa wheat proteins was associated with increased risk of diabetes (Table 2). We detected the 46+52 kDa band pattern in 82% of 50 d pre-diabetic animals (9/11) but in none of the BBc rats (0/10, p=0.0002). Similarly at 70 d, 91% of pre-diabetic animals (10/11) but none of the BBc rats (0/10, p=0.000001) showed antibody binding to the 46+52 kDa band pattern.

Table 2: Frequency of signal detected against combinations of WG proteins in pre-diabetic and asymptomatic BBdp and BBc rats (84F)

Bands	Comparison	Age	BBc n=10 n(%)	BBdp asympt n=3 n(%)	BBdp pre-T1D n=11 n(%)	Band pattern p-value
38+33	pre-T1D vs asympt.	50d	n/a	0 (0)	3 (27)	0.45
36+33	pre-T1D vs asympt.	50d	n/a	0 (0)	7 (64)	0.096
38+36+33	pre-T1D vs asympt.	50d	n/a	0 (0)	3 (27)	0.45
38+33	control vs pre-T1D	50d	0 (0)	n/a	3 (27)	0.12
36+33	control vs pre-T1D	50d	0 (0)	n/a	7 (64)	0.0028
38+36+33	control vs pre-T1D	50d	0 (0)	n/a	3 (27)	0.12
38+33	pre-T1D vs asympt.	70d	n/a	1(33)	1(9)	0.39
36+33	pre-T1D vs asympt.	70d	n/a	1(33)	4(36)	0.72
38+36+33	pre-T1D vs asympt.	70d	n/a	1(33)	1(9)	0.39
38+33	control vs pre-T1D	70d	0 (0)	n/a	1(9)	0.52
36+33	control vs pre-T1D	70d	0 (0)	n/a	4(36)	0.055
38+36+33	control vs pre-T1D	70d	0 (0)	n/a	1(9)	0.52
52+46+40+38+36+33	pre-T1D vs asympt.	50d	n/a	0 (0)	2(18)	0.6
46+40+38+36+33	pre-T1D vs asympt.	50d	n/a	0 (0)	2(18)	0.6
40+36+33	pre-T1D vs asympt.	50d	n/a	0 (0)	5(45)	0.23
52+46	pre-T1D vs asympt.	50d	n/a	1(33)	9(82)	0.17
52+46+40+38+36+33	control vs pre-T1D	50d	0 (0)	n/a	2(18)	0.26
46+40+38+36+33	control vs pre-T1D	50d	0 (0)	n/a	2(18)	0.26
40+36+33	control vs pre-T1D	50d	0 (0)	n/a	5(45)	0.023
52+46	control vs pre-T1D	50d	0 (0)	n/a	9(82)	0.0002
52+46+40+38+36+33	pre-T1D vs asympt.	70d	n/a	0 (0)	0 (0)	1
46+40+38+36+33	pre-T1D vs asympt.	70d	n/a	0 (0)	0 (0)	1
40+36+33	pre-T1D vs asympt.	70d	n/a	0 (0)	0 (0)	1
52+46	pre-T1D vs asympt.	70d	n/a	3(100)	10(91)	1
52+46+40+38+36+33	control vs pre-T1D	70d	0 (0)	n/a	0 (0)	1
46+40+38+36+33	control vs pre-T1D	70d	0 (0)	n/a	0 (0)	1
40+36+33	control vs pre-T1D	70d	0 (0)	n/a	0 (0)	1
52+46	control vs pre-T1D	70d	0 (0)	n/a	10(91)	0.000001

pre-T1D: pre-diabetic
asympt.: asymptomatic

The combination of 33+36 kDa WG bands was also more frequently detected in 50 d pre-diabetic BBdp rats (7/11, 64%) compared with BBc animals (0/10, p=0.003). Fewer 70 d pre-diabetic (4/11, 36%) reacted to this band pattern and no reactivity was detected in 70 d BBc rats (p=0.06).

In the 86F study, 15 BBdp animals were weaned onto the WG diet of which 11 rats developed diabetes, while 4 were asymptomatic. Western blot analysis showed that blood samples taken at 50d from asymptomatic and pre-diabetic BBdp rats did not show significant differences in the antibody binding (Figure 10, Panel A). No antibody binding was detected to 54-140 kDa WG proteins. The strongest signal was detected to 50 and 52 kDa proteins (0.3-0.4 OD). These bands were detected in all 50 and 70 d BBdp rats (Figure 11, Panel A). At 70 d, prediabetic rats showed higher IgG reactivity to 38 kDa (p=0.008) and 36 kDa (p=0.04) compared with asymptomatic rats (Figure 10, Panel B). All prediabetic animals (n=11) reacted to 38 kDa proteins, while half of the asymptomatic animals (2/4) had detectable antibody binding (p=0.05). A similar frequency of BBdp rats in both risk groups reacted to 36 kDa proteins (6/11 (55%) prediabetic and 1/4 (25%) asymptomatic; p= 0.6).

We examined various combinations of protein bands to determine if reactivity was associated with increased risk of disease (Table 3). At 50 d, a similar proportion of pre-diabetic and asymptomatic BBdp rats responded to all combinations of wheat gluten proteins. The concomitant presence of the 33, and 38 kDa bands at 70 days was most frequently associated with diabetes. We detected this band pattern in 10/11 (91%) pre-diabetic animals and 1/4 (25%) asymptomatic rats at 70 days (p=0.03).

Figure 10 Western blot of wheat gluten proteins probed with serum samples taken at 50 and 70 days from BBdp rats fed WG (86F).

Serum samples from pre-diabetic (n=11) and asymptomatic (n=4) BBdp rats fed a WG diet were taken at 50 days (Panel A) and 70 days (Panel B) from each animal. Western blots of wheat gluten proteins extracted with 9M urea lysis buffer wereprobed with individual serum samples. IgG antibody binding was detected using an anti-rat total IgG antibody labeled with HRP, which was visualized by enhanced chemiluminescence. Mean absorbance $\pm$ SD of each wheat gluten protein band is shown in bar graphs. Representative blots are shown on the right. ANOVA post hoc comparisons of means using LSD test of 70d pre-diabetic and asymptomatic BBdp rats; § indicates p=0.008, ✱ indicates p=0.04.

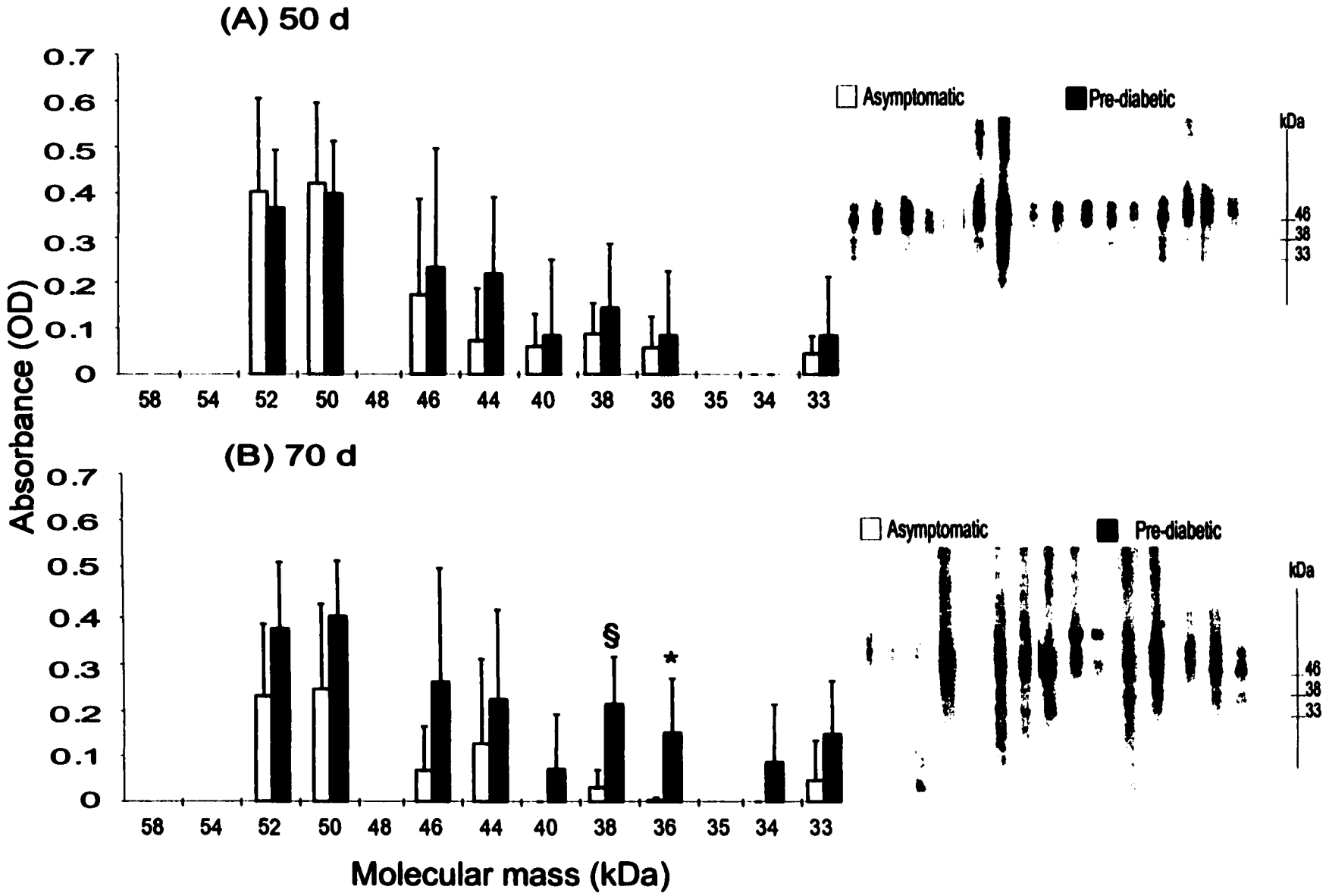

(A) 50 d
Asymptomatic
Pre-diabetic
kDa
46
38
33
Absorbance (OD)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
58 54 52 50 48 46 44 40 38 36 35 34 33
(B) 70 d
Asymptomatic
Pre-diabetic
kDa
46
38
33
§
*
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
58 54 52 50 48 46 44 40 38 36 35 34 33
Molecular mass (kDa)

Figure 11 Frequency of 50 and 70 day old BBdp rats with IgG antibodies to wheat gluten proteins (86F).

Serum samples from pre-diabetic (n=11) and asymptomatic (n=4) BBdp rats fed a WG diet were taken at 50 days (Panel A) and 70 days (Panel B) from each animal. Western blots of wheat gluten proteins extracted with 9M urea lysis buffer were probed with individual serum samples. IgG antibody binding was detected using an anti-rat total IgG antibody labeled with HRP, which was visualized by enhanced chemiluminescence.

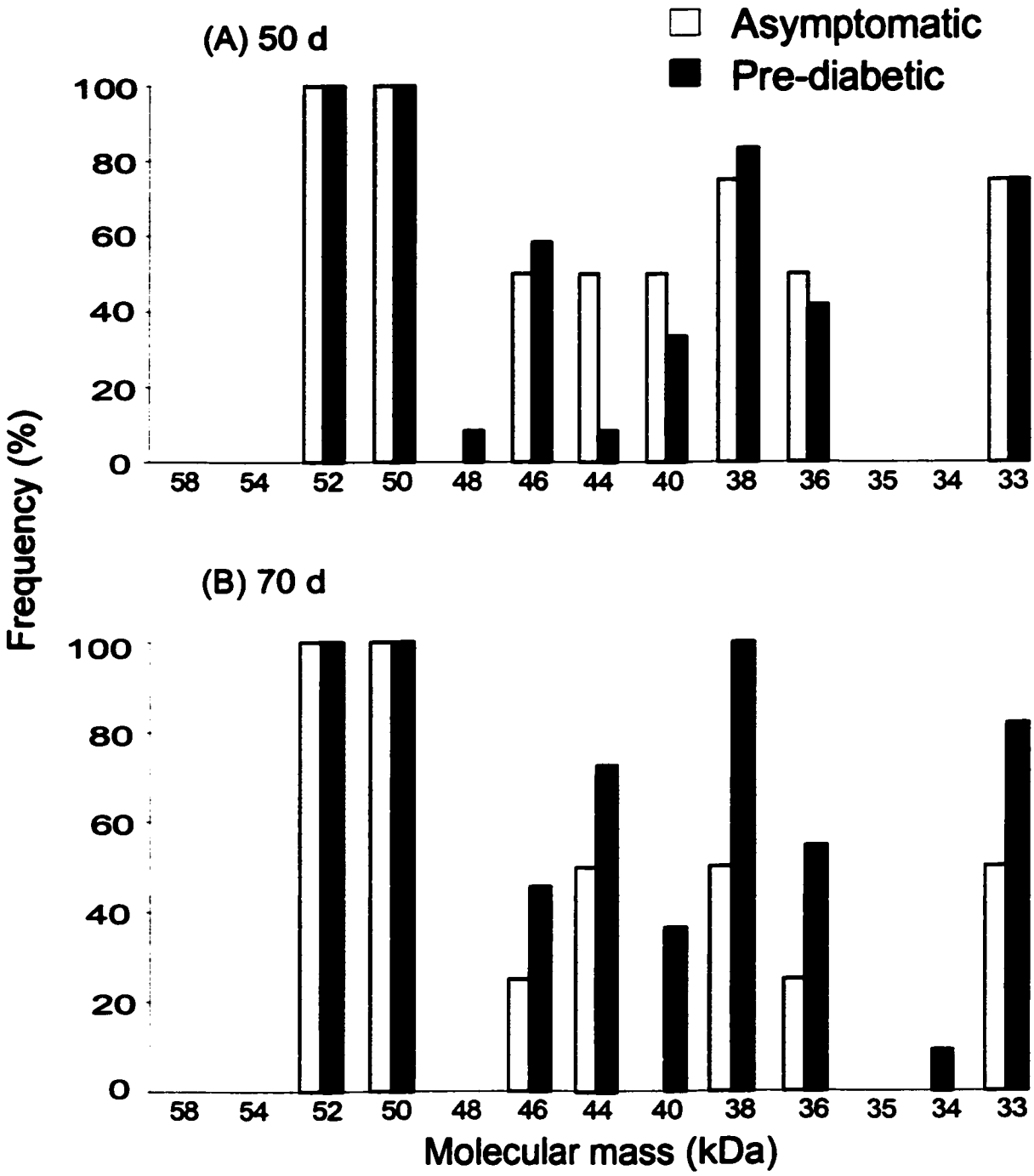

(A) 50 d
Asymptomatic
Pre-diabetic
100
80
60
40
20
0
58
54
52
50
48
46
44
40
38
36
35
34
33
(B) 70 d
Frequency (%)
Molecular mass (kDa)

Table 3: Frequency of signal detected against combinations of WG proteins in pre-diabetic and asymptomatic BBdp and BBc rats (86F)

Band pattern kDa	Comparison	Age	BBdp asympt. n=4 n (%)	BBdp pre-T1D n=11 n (%)	Band pattern p-value
33+36+38+40+44+46	Asympt. vs pre-T1D	50d	0 (0)	3 (27)	0.41
33+36+38+44+46	Asympt. vs pre-T1D	50d	0 (0)	3 (27)	0.41
33+36+38+46	Asympt. vs pre-T1D	50d	2 (50)	4 (36)	0.53
33+36+46	Asympt. vs pre-T1D	50d	2 (50)	4 (36)	0.53
33+36+38	Asympt. vs pre-T1D	50d	2 (50)	4 (36)	0.53
33+38	Asympt. vs pre-T1D	50d	3 (75)	6 (54)	0.46
33+36	Asympt. vs pre-T1D	50d	2 (50)	4 (36)	0.53
36+38	Asympt. vs pre-T1D	50d	2 (50)	5 (45)	0.66
33+36+38+40+44+46	Asympt. vs pre-T1D	70d	0 (0)	2 (18)	0.52
33+36+38+44+46	Asympt. vs pre-T1D	70d	0 (0)	4 (36)	0.24
33+36+38+46	Asympt. vs pre-T1D	70d	0 (0)	5 (45)	0.15
33+36+46	Asympt. vs pre-T1D	70d	0 (0)	4 (36)	0.24
33+36+38	Asympt. vs pre-T1D	70d	0 (0)	7 (64)	0.051
33+38	Asympt. vs pre-T1D	70d	1 (25)	10 (91)	0.033
33+36	Asympt. vs pre-T1D	70d	0 (0)	7 (64)	0.051
36+38	Asympt. vs pre-T1D	70d	1 (25)	8 (73)	0.14

pre-T1D: pre-diabetic
Asympt.: asymptomatic

We also observed a trend in the reactivity to 33+36 kDa wheat proteins, which were detected in 64% (7/11) pre-diabetic animals but in none of the asymptomatic rats (0/4, p=0.05). The identity of these antigenic proteins cannot be deduced solely from molecular mass analysis because each protein band is a heterogeneous mixture of several proteins (i.e. gliadins and glutenins co-migrate on polyacrylamide gel). Therefore, the antigenic wheat proteins were characterised in more detail by 2 dimensional electrophoresis, Western blotting and mass spectrometry to obtain their amino acid sequence and identify them by searching homologous sequences in the NCBI database.

Study #4: 2D Western blots

The 33, 36 and 38 kDa wheat proteins were identified as potential prognostic markers of diabetes development in Study #3. Because the identity of these (and other) proteins cannot be deduced from their respective molecular mass alone, our aim was to characterise antigenic WG proteins by proteomic and Western blot analysis. Wheat proteins were separated by two-dimensional electrophoresis and the greater resolution permitted identification of several hundred discrete proteins, which were probed using serum antibodies applied to Western blots. Antigenic proteins were excised for mass spectrometry analysis to obtain the amino acid sequence. This approach has been used before by others to identify wheat flour allergens [125].

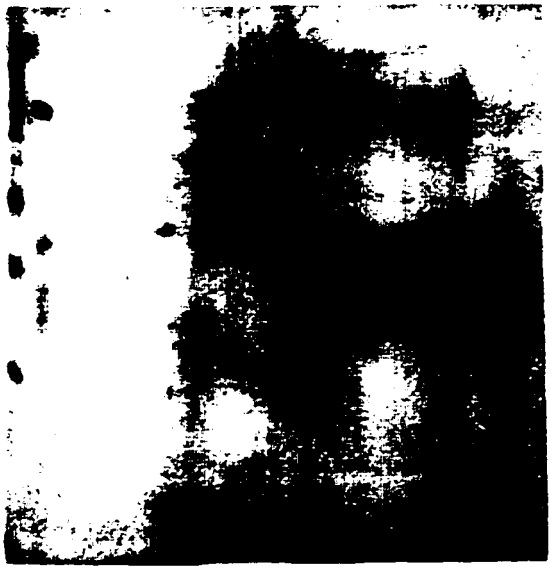
Two-dimensional gel electrophoresis consisted of isoelectric focusing (IEF) in the first dimension and sodium dodecyl sulphate, polyacrylamide gel electrophoresis (SDS-PAGE) in the second dimension. Serially diluted WG proteins were separated on 2D gels (Figure 12). We detected with a silver stain at least 300 discrete wheat proteins in 150 μg of protein extract.

Figure 12 Two dimensional electrophoresis of wheat gluten proteins stained with silver

A serial dilution of 9M urea wheat gluten protein extract (38, 75, 150 and 300 μg) was fractionated by 2-dimensional electrophoresis. In the first dimension, proteins were separated by isoelectric focusing on pH 3-10 immobilized linear pH gradient strips. In the second dimension, proteins were separated by size on a 10% sodium-dodecyl sulphate, polyacrylamide gel electrophoresis. The proteins were detected by silver stain.

(A) 38 μg

(B) 75 μg



(C) 150 μg

(D) 300 μg

2D-gels of 150 μg of WG protein were blotted onto pure nitrocellulose membrane for Western blot analysis. We probed these 2D blots with prospective serum samples that were taken at day 50, 70 and at necropsy from individual BBdp rats fed a WG diet. The serum samples were classified retrospectively as asymptomatic or pre-diabetic based on the diagnosis of diabetes by 120 days (n=5 BBdp rats that went on to develop diabetes at 80-100 days; n=5 BBdp rats that remained asymptomatic by 120 days). Each 2D blot was probed with serum from one animal from one of the sample time points. In total our database consisted of 30 2D blots: 5 animals/group x 2 groups (asymptomatic and pre-diabetic) x 3 time points (50 d, 70 d and necropsy). An image of each blot was captured on the Kodak image station and analysed using PDQuest 2D image analysis software. The software aligned the thirty 2D blots using landmarks proteins (3-5 immunogenic proteins present on all gels). The same detection parameters were applied to all images to locate the immunogenic proteins. The software assigned a preliminary reference number for each immunogenic WG protein. The data showed that 3 out of 5 BBdp rats had IgG antibodies to 11 wheat gluten proteins (Table 4, WG# 4508, 4603, 4604, 4703, 5406, 5501, 5503, 5504, 5505 and 5701). The most frequently immunogenic proteins in pre-diabetic animals compared with asymptomatic rats were WG# 4508 (80% vs 40%). WG# 5501 was detected in all asymptomatic animals (n=5) but only in 40% of pre-diabetic rats (2/5).

Figure 13 shows the mean intensity of the signal detected for 11 antigenic WG proteins. The mean reactivity to WG# 5505 was higher in asymptomatic animals than pre-diabetic rats at 70 days (p=0.04).

Figure 13 Mean signal intensity detected on 2D Western blot of wheat gluten proteins probed with sera from 50, 70 and 120 days old BBdp rats fed WG

9M urea extract of wheat gluten proteins was separated by 2D SDS-PAGE and probed with prospective serum samples from BBdp rats fed WG. Serum samples obtained at 50, 70 and at diagnosis of diabetes or 120 days (asymptomatic). IgG antibody binding was detected using an anti-rat IgG antibody labeled with HRP, which was detected by enhanced chemiluminescence. Mean absorbance $\pm$ SD of wheat gluten proteins is shown in bar graphs. Data were analyzed by ANOVA and post hoc comparisons of means using LSD test. For WG proteins #5505; asymptomatic 70 d animals had higher IgG binding compare with 70 d pre-diabetic rats, ★ indicates p=0.04. At WG protein #5701; diabetic animals had higher IgG binding than 120 day old asymptomatic animals, ‡ indicates p=0.03.

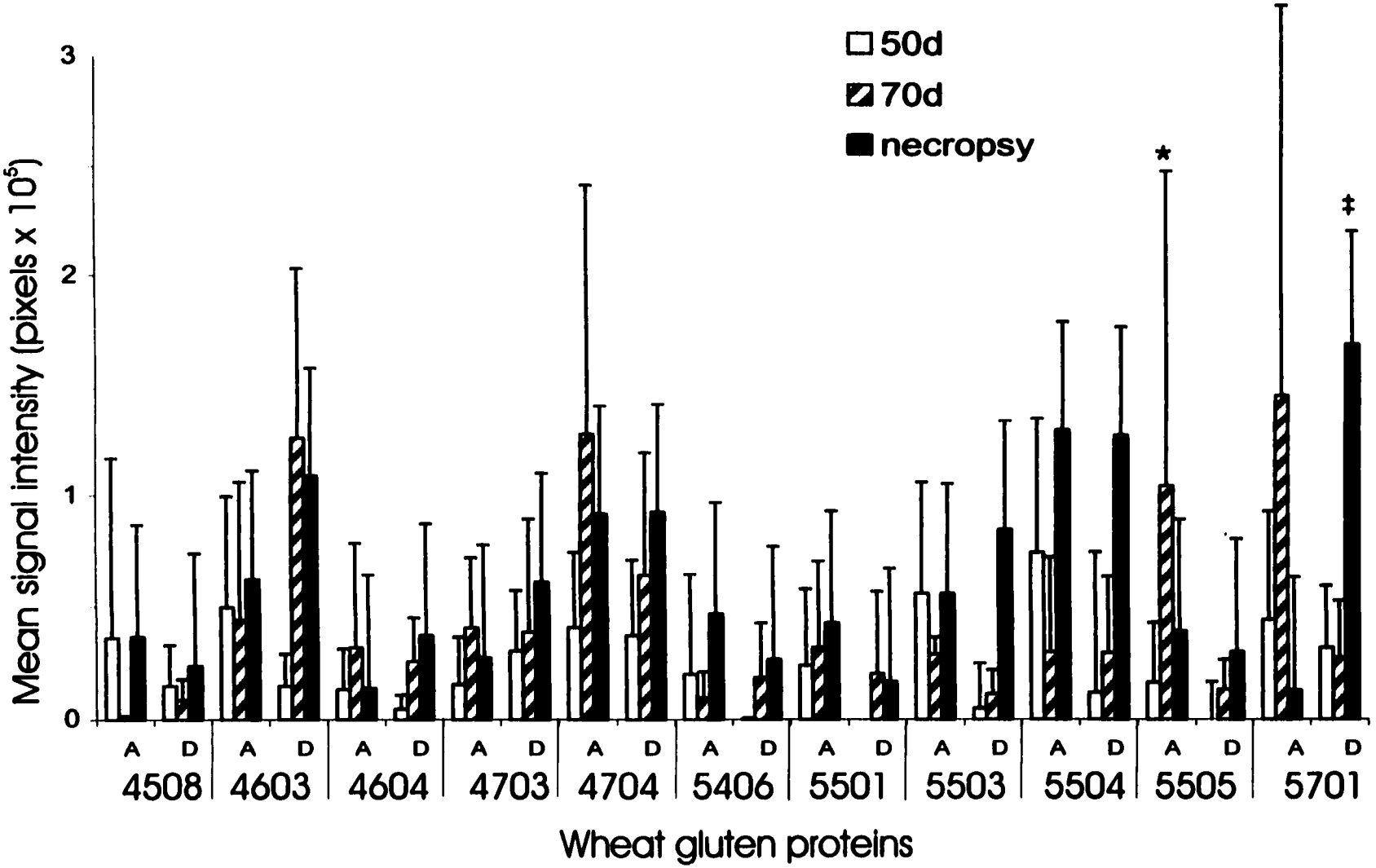

50d
70d
necropsy
Mean signal intensity (pixels x 10⁵)
0
1
2
3
*
‡
A D A D A D A D A D A D A D A D A D A D
4508 4603 4604 4703 4704 5406 5501 5503 5504 5505 5701
Wheat gluten proteins

Table 4: Frequency of BBdp rats with serum IgG antibodies to WG peptides detected by 2D Western blot

WG peptides	Rat A	B	C	D	E	F	G	H	I	J	Asympt (%)	pre-T1D (%)	Total (%)
4508		OX			X	X	XO		X	O	40	80	60
4603		O	OX	OX	X	OX	X	X	OX	OX	80	100	90
4604		O	OX	OX	OX	OX		X	OX	X	80	80	80
4703		X	OX	OX	X	OX	X		O	OX	80	80	80
4704		OX	OX	OX	OX	OX		X	OX	OX	80	80	80
5406			X	X	X		X			O	60	40	50
5501	OX	OX	X	O	X	X	OX				100	40	70
5503	OX	OX	OX	OX	OX	OX	OX		OX	X	100	80	90
5504	O	O	OX	OX	OX	OX	OX		OX	X	100	80	90
5701		OX	OX	OX	X	OX		X	OX	OX	80	80	80
5505			OX	OX				X	OX	X	40	60	50

Legend:
A-E: asymptomatic BBdp rats
F-J: pre-diabetic BBdp rats
O indicates antibody binding at 50 days detected
X indicates antibody binding at 70 days detected
Asympt.: asymptomatic BBdp rats
Pre-T1D: pre-diabetic BBdp rats

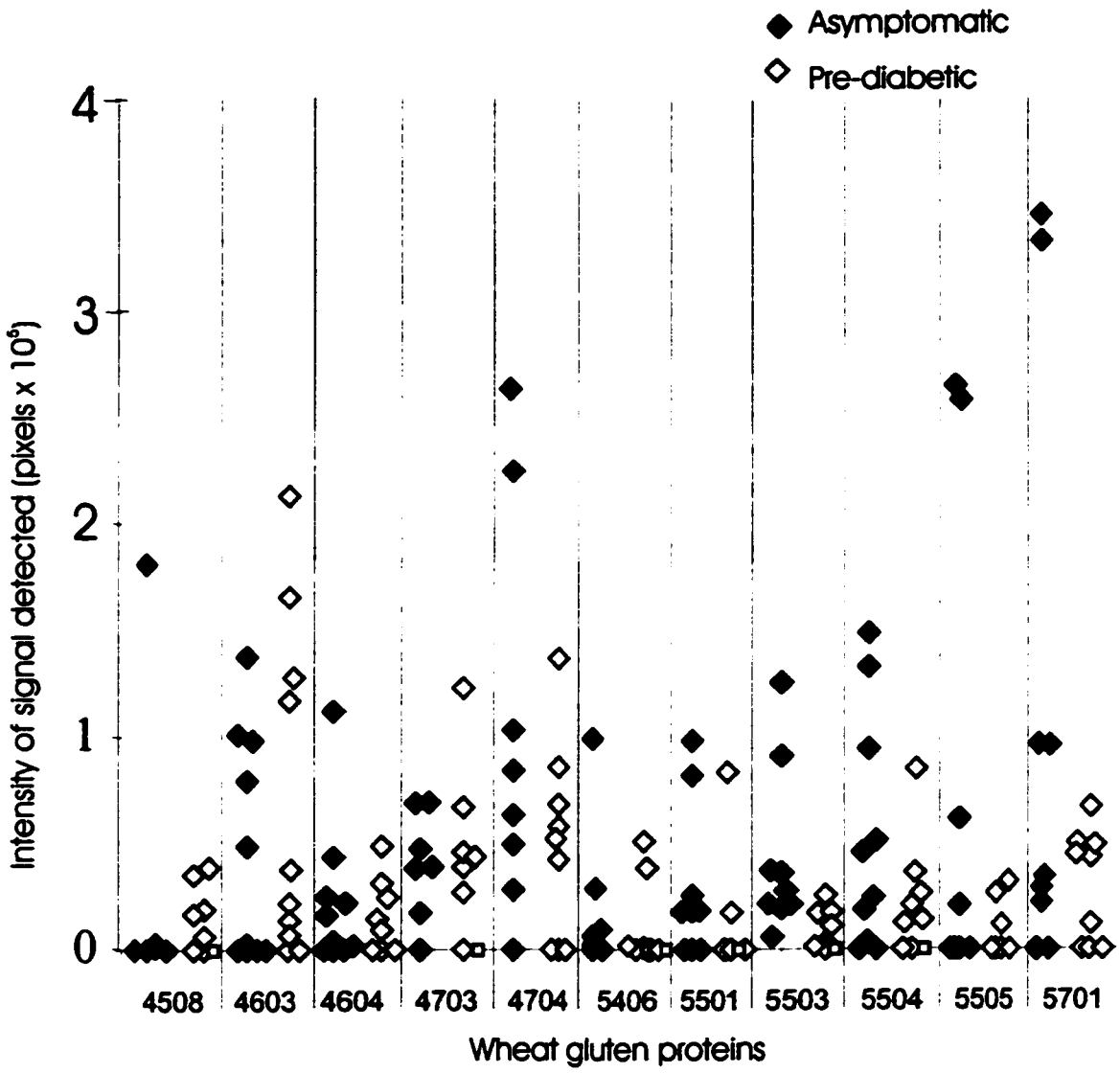
In contrast, the mean signal detected for WG# 5701 was higher in diabetic animals than 120 day old asymptomatic animals at necropsy ($p=0.03$). A scatter plot of the signal intensity detected at 50 or 70 days for the 11 wheat proteins showed several more trends (Figure 14). There was a low level of IgG binding to WG# 4508 in asymptomatic animals and a trend toward higher reactivity was detected in pre-diabetic animals. The same trend was observed at WG# 4603. The level of antibody binding to WG# 4604, 4703, 4704, 5406, 5501, 5503 and 5504 was similar in pre-diabetic and asymptomatic animals. Mass spectrometry analysis of all 11 proteins was performed to obtain the amino acid sequence of these proteins and identify them from published databases.

Study #5: Identification of immunogenic wheat proteins

The 2D Western blots in Study #4 showed that the majority of BBdp rats have IgG antibody reactivity to 11 WG proteins. In order to determine the identity of these proteins we collaborated with Drs John Kelly and Pierre Thibault at the National Research Council (NRC) to perform the mass spectrometry analysis. A serial dilution of the urea extract of WG proteins, featuring 38 μg, 75 μg, 150 μg and 300 μg of proteins was resolved on four- 2D gels to determine the optimal resolution for each of the protein spots (Figure 12). The gels were stained with silver to detect proteins and the immunogenic spots were located based on several landmark proteins that were detected on the 2D Western blots. Replicate samples of the proteins of interest were excised from multiple gels for amino acid sequence analysis. We also included a known protein for MS/MS analysis as a positive control.

Figure 14 Signal intensity detected on 2D Western blot of wheat gluten proteins probed with sera from 50, 70 and 120 day old BBdp rats fed WG

9M urea extract of wheat gluten proteins was separated by 2D SDS-PAGE and probed with prospective serum samples from BBdp rats fed WG. Serum samples obtained at 50 , 70 and at diagnosis of diabetes or 120 days (asymptomatic). IgG antibody binding was detected using an anti-rat IgG antibody labeled with HRP, which was visualized by enhanced chemiluminescence.


Asymptomatic
Pre-diabetic
Intensity of signal detected (pixels x 10⁵)
4
3
2
1
0
4508
4603
4604
4703
4704
5406
5501
5503
5504
5505
5701
Wheat gluten proteins

The excised gel pieces containing the proteins of interest were digested with trypsin, the peptides were extracted with organic buffers, the peptide fragments were separated by liquid capillary electrophoresis and the amino acid sequence was obtained by mass spectrometric analysis. We obtained amino acid sequences for 5 of the 11 proteins sent for analysis at the NRC (Table 5). The remaining proteins did not yield sequence information. One explanation may be that the chemical structure of some WG proteins, makes them inaccessible to the enzyme. Previous reports showed that digestion with pepsin, trypsin or pancreatin also yielded appreciable amounts of undigested proteins [126].

The train of 4 immunogenic proteins WG#4508, WG#5501, WG#5503 and WG#5504 were identified by MS/MS analysis as alanine aminotransferase (Glutamic-pyruvic transaminase 2 derived from barley, GPT-2, Accession No. P52894). The immunogenic peptides were identified as GPT-2 based on 10 peptide fragments detected by mass spectrometry (Table 5a) that were compared to a theoretical trypsin digest of barley GPT-2 [79]. All 10 protein fragments matched the theoretical trypsin digest of barley GPT-2. Although, the NCBI database is the largest compendium of publicly available protein sequences, it does not contain all known wheat protein sequences. Therefore, we searched a wheat genome database, The TIGR *Triticum aestivum* Gene Index [127] to find a wheat alanine aminotransferase 2 transaminase 2 (TC# 8197). All 10 protein fragments detected by MS/MS following trypsin digestion matched the expected protein fragments after a theoretical trypsin digest of wheat GPT-2 (Table 5a). The experimental molecular mass of WG#4508, WG#5501, WG#5503 and WG#5504 was 65 kDa, which is close to the theoretical molecular mass of wheat GPT-2, 64 kDa.

Table 5a: Peptides detected following tryptic digest of GPT-2

Protein	Theoretical fragmentation	Experimental fragmentation
GPT-2 barley P52894 pI: 5.93 Mw: 53 kDa	ALVVINPGNPTGQVLAEENQYDIVK(9) LEGITCNEAEGAMYVFPQICLPQK LLESTGIVVVPGSGFGQVPGTWHFR IASVNLCSNITGQILASLVMNPPK NEGLVLLADEVYQENIYVDNK SLGYGEEDLPLVSYQSVSK (10) FTVFHEAFMSEYR EVLALCDHPDLLQR GGYFEITGFSAPVR MAATVAVDNLNPK ATGAYSHSQGIK (5) ASDESYASYK TLFSADSISR (4) CTILPQEDK APDAFYALR (6) GEIVIHAQR (3) QILAMIPGR (2) DAIASGIASR (1) ALEHAFNK DGILASLAR GYYGECGK LQEQLK IPAVISR CEYAVR QLEDAR EQIYK	DAIASGIASR (1) QILALIPGR (2) GEIVIHAQR (3) TLFSADSISR (4) ATGAYSHSQGIK (5) APDAFYALR (6) DGILASLAR (7) ASDESYASYK (8) ALVVINPGNPTGQVLAEENQYDIVK(9) SLGYGEEDLPLVSYQSVSK (10)
GPT-2 Wheat wheat|TC 8197 pI: 6.33 Mw: 64 kDa	ALVVINPGNPTGQVLAEENQYDIVK LLESTGIVVVPGSGFGQVPGTWHFR NEGLVLLADEVYQENIYADNK SLGYGEEDLPLVSYQSVSK (10) AEGAMETYLFPQICLPQK FTVFHETFMETAEYR GGYMETEITGFSAPVR EVLALCDHPDLLK ATGAYSHSQGIK (5) ASDESYASYK TLFSADSISR (4) HIAFLSPFR CTILPQEEK APDAFYALR (6) GEIVIHAQR (3) QILALIPGR (2) DAIASGIASR (1) DGILASLAR LEGITCNK GYYGECGK ALEVAFSK IPAIISR CEYAVR SLLLYK QLEDAR	DAIASGIASR (1) QILALIPGR (2) GEIVIHAQR (3) TLFSADSISR (4) ATGAYSHSQGIK (5) APDAFYALR (6) DGILASLAR (7) ASDESYASYK (8) ALVVINPGNPTGQVLAEENQYDIVK(9) SLGYGEEDLPLVSYQSVSK (10)

Table 5b: Peptides detected following tryptic digest of GB-1 and wheat storage protein

Protein	Theoretical fragmentation	Experimental fragmentation
GB-1 Barley S35221 Consists of four subunits pI: 6.80 Mw: 72 kDa	AQDQDEGFVAGPEQQSR FQFLSVKPLLASLSK GSESESEEEEEQQR LGSPAQELTFGRPAR DTFNLLEQRPK SFHALANQDVR GGHSLQQCVQR ALRPFDQVSR IIQSDHGFVR HEQEEEQGR GDEAVETFLR EQEQEQER ILHTISVPGK EEEEDDQR VAIMEVNPR (11) EAAEGGQGHR RPYVFGPR (12) DDQQQHGR DEEQGDSR GWHGEGER WPLPPFR EVQEVFR	RPYVFGPR (11) VAIMEVNPR (12) LWQIPEQSR
Wheat storage protein - globulin AAA34269 Consists of two subunits: pI: 6.55 6.90 Mw: 49kDa 35 kDa	AQDQDEGFVAGPEQQSR FQFLSVKPLLASLSK GSESESEEEEEQQR LGSPAQELTFGRPAR DTFNLLEQRPK SFHALANQDVR GGHSLQQCVQR ALRPFDQVSR IIQSDHGFVR HEQEEEQGR GDEAVETFLR EQEQEQER ILHTISVPGK EEEEDDQR VAIMEVNPR (11) EAAEGGQGHR RPYVFGPR (12) DDQQQHGR DEEQGDSR GWHGEGER WPLPPFR EVQEVFR	RPYVFGPR (11) VAIMEVNPR (12) LWQIPEQSR

We did not use any marker proteins to determine the isoelectric points (pI) because the marker proteins would have co-migrated with wheat proteins, which would confound our 2D blot analysis. The acidic (pI 3) and basic (pI 10) ends of the IPG strips are fixed and there is a linear gradient from pI 3 to 10. Interpolation of the pI values between pI 3-10 showed that GPT-2 in our 2D gels had a pI of 6.5. The peptide fragments of WG #4508, 5501, 5503 and 5504 digested with trypsin and detected by mass spectrometry are demarcated on the full sequence of GPT-2 in Table 6. There were no significant differences in the level of reactivity to GPT-2 in sera from asymptomatic, pre-diabetic and diabetic BBdp rats (Figure 15). When we probed a WG 2D blot with pooled sera from newly diagnosed Finnish children and non-diabetic control children, reactivity to GPT-2 proteins (WG #4508, 5501, 5503 and 5504) was detected in sera from children newly diagnosed with diabetes but no IgG binding was detected in non-diabetic HLA-DQ matched control children (Figure 16).

The peptide sequences derived from spot WG#5406 were identified as globulin proteins by MS/MS analysis and queries of the NCBI database. MS/MS analysis showed 2 peptide fragments following trypsin digestion that matched the theoretical tryptic digest of barley globulin Beg1 precursor (barley GB-1, Acc. No. S35221) and that of wheat storage globulin (WSG, Acc. No. AAA34269, Table 5b). The source of the protein in our 2D gel analysis was wheat and therefore the candidate proteins isolated from the 2D gel is a wheat storage globulin (WSG, Acc. No. AAA34269). WG #5406 had a molecular mass of 50 kDa on our 2D blots, which is close to the expected molecular mass of 49 kDa for WSG [128].

Figure 15 Mean signal intensity of wheat storage globulin and alanine aminotransferase-2 proteins detected on 2D Western blots.

9M urea extract of wheat gluten proteins was separated by 2D SDS-PAGE and probed with prospective serum samples from 50, 70 day and necropsy BBdp rats fed WG. IgG antibody binding was detected using an anti-rat IgG antibody labeled with HRP, which was detected by enhanced chemiluminescence. Mean signal intensity of wheat storage globulin (Acc. No. AAA34269) and alanine aminotransaminase-2 proteins (GPT-2, Acc. No. TC8197) detected on 2D Western blots probed with serum from 50, 70 day or necropsy (n=5) BBdp rats.

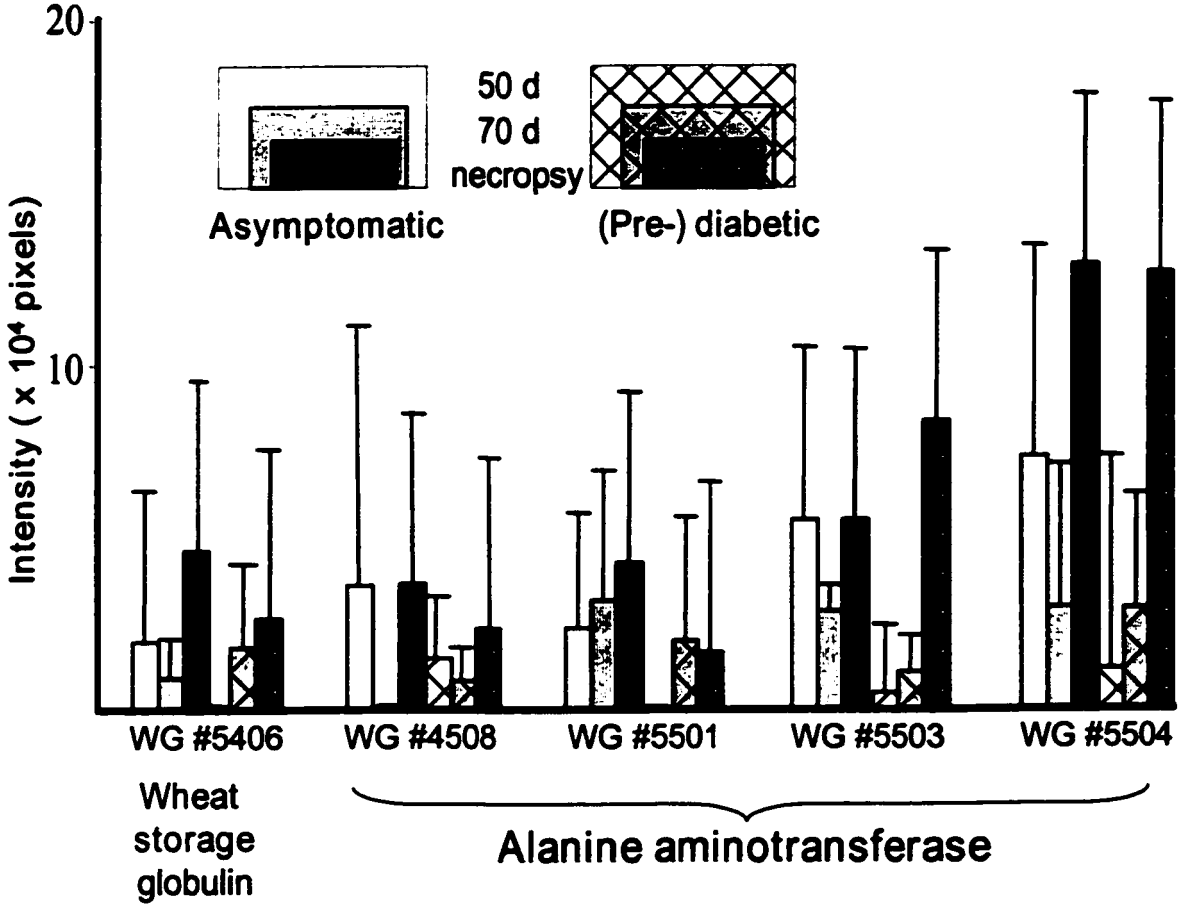
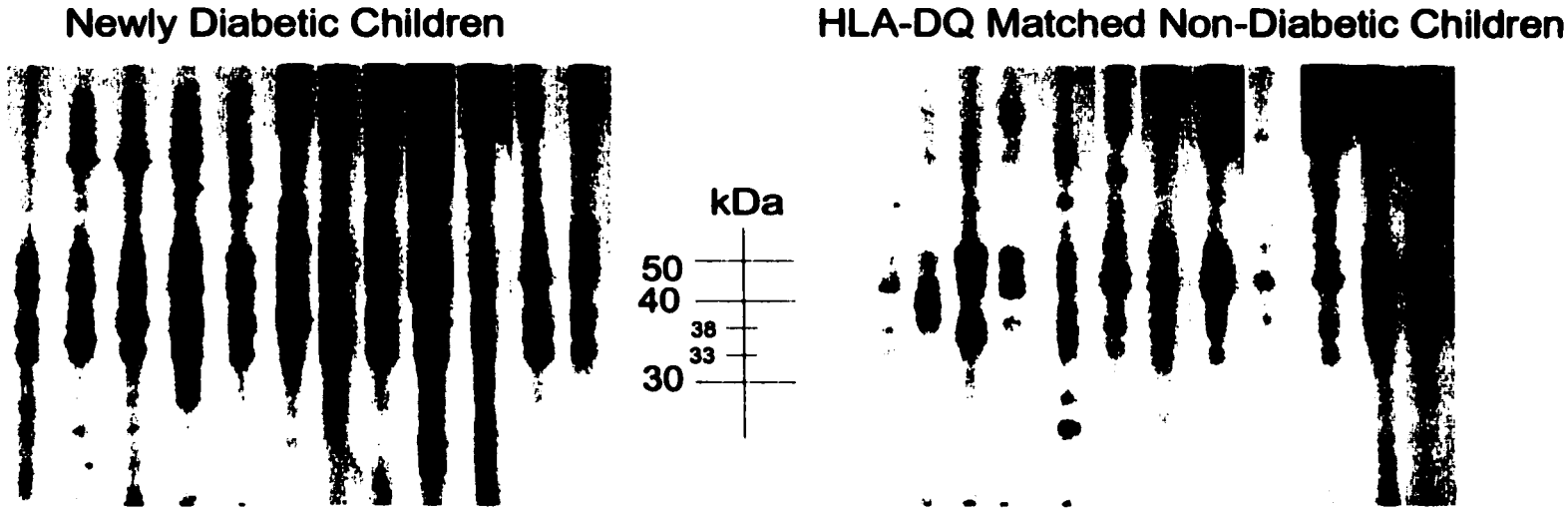

20
10
Intensity (x 10⁴ pixels)
50 d
70 d
necropsy
Asymptomatic
(Pre-) diabetic
WG #5406
WG #4508
WG #5501
WG #5503
WG #5504
Wheat storage globulin
Alanine aminotransferase

Figure 16 1D and 2D Western blot of wheat gluten proteins probed with sera from newly diagnosed Finnish children and HLA-DQ and age matched (non-diabetic) control children.

1D Western blot: serum samples from newly diagnosed diabetic Finnish children and control children were used at a dilution of 1:50 to probe wheat gluten proteins extracted with 9M urea lysis buffer. IgG antibody binding was detected using anti-human total IgG antibody labeled with HRP and visualized by enhanced chemiluminescence on film.

2D Western blot: pool of serum from newly diagnosed diabetic Finnish children or control children were used at a dilution of 1:500 to probe wheat gluten proteins extracted with 9M urea lysis buffer. IgG antibody binding was detected using anti-human total IgG antibody labeled with HRP and visualized by enhanced chemiluminescence on film.

1D Western blots



2D Western blots

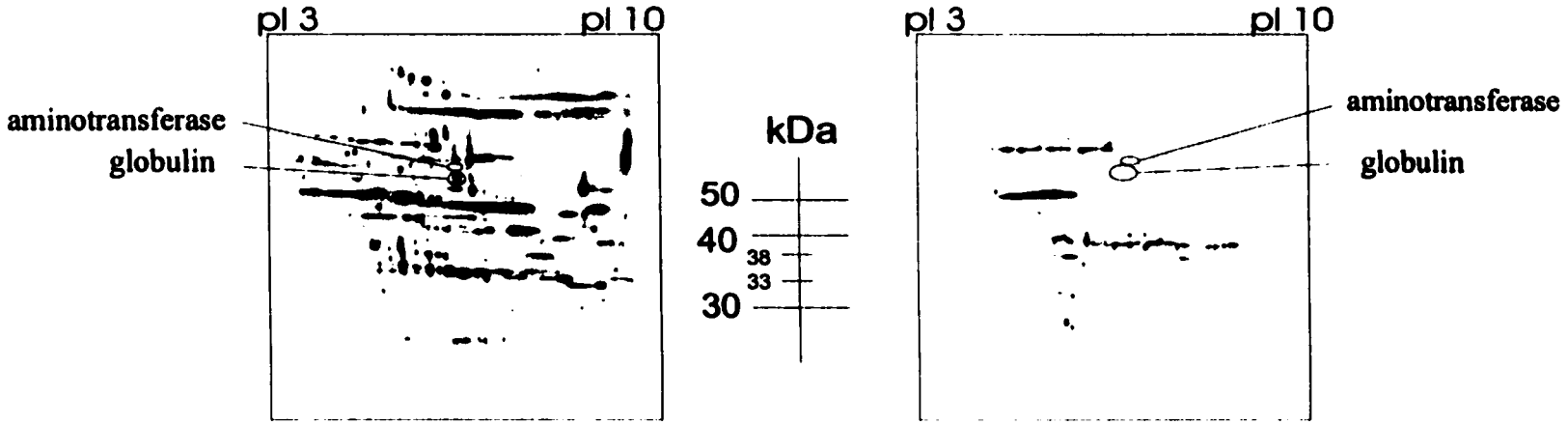


Table 6a: Wheat gluten protein sequences determined by mass spectrometry and closest homology (barley GPT 2)

Gel spot	Peptide sequences	Closest Homology
4508	DAIASGIASR (1) QILALIPGR (2) GEIVIHAQR (3) TLFSADSISR (4) ATGAYSHSQGIK (5) APDAFYALR (6)	**Alanine amino-transferase 2** (GPT-2, barley) P52894 gi|1703227|sp|P52894|ALA2_HORVU (3) MAATVAVDNL NPKVLKCEYA VRGEIVIHAQALLQEQLKTQP GSLPFDEILY CNIGNPQSLG (4) (2) (5) QQPVTFFREV LALCDHPDLL QREEIKTLFSADSISRAKQTLQAMLEGFVTQAYSHSQGIKG (1) LRDAIASGIASRDGFPANAD DIFLTDGASP GVHLMMQLLI RNEKDGILVP IPQYPLYSAS IALHGGALVP YYLNESTGWG LETSDVKKQL EDARSRGINV RALVVINPGN PTGQVLAEEN QYDIVKFCKN EGLVLLADEV YQENIYVDNK KFHSFKKIVR SLGYGEEDLP LVSYQSVSKG YYGECGKRGG YFEITGFSAP VREQIYKIAS VNLCSNITGQ ILASLVMNPP KASDESYASY KAEKDGILAS LARRAKALEH AFNKLEGITC NEAEGAMYVF PQICLPQKAI EAAKAANKAP (6) DAFYALRLLE STGIVVVPGS GFGQVPGTWH FRCTILPQED KIPAVISRFT VFHEAFMSEY RD
5501	DAIASGIASR (1) QILALIPGRG (2) GEIVIHAQR (3) ATGAYSHSQGIK (5) APDAFYALR (6)	**Alanine amino-transferase 2** (GPT-2, barley) P52894 gi|1703227|sp|P52894|ALA2_HORVU See above

Table 6b: continued (barley GPT-2)

5503	DAIASGIASR (1) GEIVIHAQR (3) TLFSADSISR (4) ATGAYSHSQGIK (5) APDAFYALR (6) DGILASLAR (7) ASDESYASYK (8) ALVVINPGNPTGQVLAEENQYDIVK (9) SLGYGEEDLPLVSYQSVSK (10)	**Alanine amino-transferase 2** (GPT-2, barley) P52894 gi|1703227|sp|P52894|ALA2_HORVU (3) MAATVAVDNL NPKVLKCEYA VRGEIVIHAQ RLQEQLKTQP GSLPFDEILY CNIGNPQSLG (4) (2) (5) QQPVTFFREV LALCDHPDLL QREEIKTLPS SADSISRAKQ LAMIPGRATG VKEHCQAIKG (1) LRDAIASGIA SRDGFPANAD DIFLTDGASP GVHLMMQLLI RNEKDGILVP IPQYPLYSAS (9) IALHGGALVP YYLNESTGWG LETSDVKKQL EDARSRGINV RALVVINPGN PTGQVLAEEN (10) QYDIVKFCKN EGLVLLADEV YQENIYVDNK KFHSFKKIVR SDGYGEEDLP LVSYQSVSKG (8) YYGECGKRGG YFEITGFSAP VREQIYKIAS VNLCSNITGQ ILASLVMNPP KAGDESYASY KAEKDGILAS LARRAKALEH AFNKLEGITC NEAEGAMYVF PQICLPQKAI EAAKAANKAP (6) DAFYALRLLE STGIVVVPGS GFGQVPGTWH FRCTILPQED KIPAVISRFT VFHEAFMSEY RD
5504	DAIASGIASR (1) TLFSADSISR (4) APDAFYALR (6) DGILASLAR (7) ASDESYASYK (8)	**Alanine amino-transferase 2** (GPT-2, barley) P52894 gi|1703227|sp|P52894|ALA2_HORVU See above

Table 6c: Wheat gluten protein sequences determined by mass spectrometry and closest homology (wheat GPT-2)

Gel spot	Peptide sequences	Closest Homology
4508	DAIASGIASR (1) QILALIPGR (2) GEIVIHAQR (3) TLFSADSISR (4) ATGAYSHSQGIK (5) APDAFYALR (6)	**alanine aminotransferase 2 transaminase 2 – (wheat GPT-2) The TIGR *Wheat* Gene Index (TaGI)** wheat|TC8197 RRPRAHPQSR GFPPLSIPPS LPVGEHLSPP PPRPLLPVL FPSSARSLLL YKHIAFLSPF (3) RVATKPRKER DGQDILAALP PPSPLVDLAM AATVAVDNL NPKVLKCEYA VR[redacted] (4) RLQQQLQTQP GSLPFDEILY CNIGNPQSLG QQPVTFFRE VLALCDHPDL LKKEEIK[redacted] (2) (5) (1) [redacted]DGFPAN ADDIFLTDGA SPGVHLMMQL LIRNEKDGIL VPIPQYPLYS ASIALHGGS LVPYYLNEST GWGLEISDVK KQLEDARSRG IDVRALVVIN PGNPTGQVLA EENQYDIVK FCKNEGLVLL ADEVYQENIY ADNKKFHSFK KIVRSLGYGE EDLPLVSYQS VSKGYYGEC GKRGGYMEIT GFSAPVREQI YKIASVNLCS NITGQILASL VMNPPKASDE SYASYKAEK DGILASLARR AKALEVAFSK (6) LEGITCNKAE GAMYLFPQIC LPQKAIEAAK AANK[redacted]LLESTG IVVVPGSGFG QVPGTWHFRC TILPQEEKIP AIISRFTVFH ETFMAEYRD
5501	DAIASGIASR (1) QILALIPGRG (2) GEIVIHAQR (3) ATGAYSHSQGIK (5) APDAFYALR (6)	**alanine aminotransferase 2 transaminase 2 – (wheat GPT-2) The TIGR *Wheat* Gene Index (TaGI)** wheat|TC8197 See above

Table 6d: continued (wheat GPT-2)

5503	DAIASGIASR **(1)** GEIVIHAQR **(3)** TLFSADSISR **(4)** ATGAYSHSQGIK **(5)** APDAFYALR **(6)** DGILASLAR **(7)** ASDESYASYK **(8)** ALVVINPGNPTGQVLAEENQYDIVK**(9)** SLGYGEEDLPLVSYQSVSK **(10)**	**alanine aminotransferase 2 transaminase 2 – (wheat GPT-2) The TIGR *Wheat* Gene Index (TaGI)** wheat|TC8197 (3) RRPRAHPQSR GFPPLSIPPS LPVGEHLSPP PPRPLLPVL FPSSARSLLL YKHIAFLSPF (3) RVATKPRKER DGQDILAALP PPSPLVDLAM AATVAVDNL NPKVLKCEYA VR[illegible] (4) RLQQQLQTQP GSLPFDEILY CNIGNPQSLG QQPVTFFRE VLALCDHPDL LKKEEIK[illegible] (2) (5) (1) SADS[illegible]RAKQ ILALIPGRAT GAYSHSQGIK GLR[illegible]DGFPAN ADDIFLTDGA SPGVHLMMQL LIRNEKDGIL VPIPQYPLYS ASIALHGGS LVPYYLNEST GWGLEISDVK (9) KQLEDARSRG IDVRALVVIN PG[illegible]QYDIVK FCKNEGLVLL ADEVYQENIY (10) ADNKKFHSFK KIVRSLGYGE ED[illegible]KGYYGEC GKRGGYMEIT GFSAPVREQI (8) YKIASVNLCS NITGQILASL VMNPPK[illegible]KAEK DGILASLARR AKALEVAFSK (6) LEGITCNKAE GAMYLFPQIC LPQKAIEAAK AANK[illegible]LLESTG IVVVPGSGFG QVPGTWHFRC TILPQEEKIP AIISRFTVFH ETFMAEYRD
5504	DAIASGIASR **(1)** TLFSADSISR **(4)** APDAFYALR **(6)** DGILASLAR **(7)** ASDESYASYK **(8)**	**alanine aminotransferase 2 transaminase 2 – (wheat GPT-2) The TIGR *Wheat* Gene Index (TaGI)** wheat|TC8197 See above

Table 6e: Wheat gluten protein sequences determined by mass spectrometry and closest homology (barley GB-1)

5406	RPYVFGPR (11) VAIMEVNPR (12) LWQIPEQSR	**globulin Beg1 precursor** (GB-1, barley) S35221 MATRAKATIP LLFLLGTSLL FAAAVSASHD DEDDRRGGHS LQQCVQRCRQ ERPRYSHARC VQECRDDQQQ HGRHEGEEEQ GRGRGWHGEG EREEEHGRGR GRHGEGEREE EHGRGRGRHG EGEREEERGR GHGRHGEGER EEERGRGRGR HGEGEREEEE GRGRGRGRGE ERDEEGGDSR (11) (12) RPYVFGPRSF RRIIQSDHGF VRALRPFDQV SRLLRGIRDY RVAIMEVNPR AFVVPGFTDA DGVGYVAGGE GVLTVIENGE KRSYTVKEGD VIVAPAGSIM HLANTDGRRK LVIAKILHTI SVPGKFQFLS VKPLLASLSK RVLRAAFKTS DERLELRFNG RQQQEKTRSV SIVRASEEQL RELRREAAEG QQQHRWPLPP FRGDSRDTFN LLEQRPKIAN RHGRLYEADA RSFHALANQD VRVAVANITP GSMTAPYLNT QSFKLAVVLE GEGEVQIVCP HLGRESESER EHGKGRRREE EEDDQRQQRR RGSESESEEE EEQQRYETVR ARVSRGSAFV VPPGHPVVEI SSSQGSSNLQ VVCFEINAER NERCWLAGRN NVIGKLGSPA GELTFGRPAR EVQEVFRAQD QDEGFVAGPE QQSREQEQEQ ERHRRRGDRG RGDEAVETFL RMATGAI

Table 6f: Wheat gluten protein sequences determined by mass spectrometry and closest homology (wheat storage globulin)

5406	RPYVFGPR (11) VAIMEVNPR (12) LWQIPEQSR	**wheat storage globulin** (WSG) AAA34269 MATRAKATIP LLFLLGTSLL FAAAVSASHD DEDDRRGGHS LQQCVQRCRQ ERPRYSHARC VQECRDDQQQ HGRHEGEEEQ GRGRGWHGEG EREEEHGRGR GRHGEGEREE EHGRGRGRHG EGEREEERGR GHGRHGEGER EEERGRGRGR HGEGEREEEE GRGRGRGRGE ERDEEGGDSR (11) (12) RPYVFGPRSF RRIIQSDHGF VRALRPFDQV SRLLRGIRDY R[redacted] AFVVPGFTDA DGVGYVAGGE GVLTVIENGE KRSYTVKEGD VIVAPAGSIM HLANTDGRRK LVIAKILHTI SVPGKFQFLS VKPLLASLSK RVLRAAFKTS DERLELRFNG RQQQEKTRSV SIVRASEEQL RELRREAAEG QQQHRWPLPP FRGDSRDTFN LLEQRPKIAN RHGRLYEADA RSFHALANQD VRVAVANITP GSMTAPYLNT QSFKLAVVLE GEGEVQIVCP HLGRESESER EHGKGRRREE EEDDQRQQRR RGSESESEEE EEQQRYETVR ARVSRGSAFV VPPGHPVVEI SSSQGSSNLQ VVCFEINAER NERCWLAGRN NVIGKLGSPA GELTFGRPAR EVQEVFRAQD QDEGFVAGPE QQSREQEQEQ ERHRRRGDRG RGDEAVETFL RMATGAI

Interpolation of the pI values on our 2D gels from pI 3 to pI 10 (linear gradient from pI 3, acidic end of strip to pI 10, basic end of strip) showed that WSG had a pI of 6.5. The two peptide fragments isolated by MS/MS analysis of the immunogenic protein WG #5406 are shown on the full sequence of WSG in Table 6f. The mean level of IgG antibody binding to peptides is shown in Figure 15. We did not detect any differences in the level of reactivity to WSG in serum samples from asymptomatic and pre-diabetic BBdp rats at 50d, 70d, and necropsy. Figure 15 also shows different levels of reactivity to the 4 GPT-2 isoforms (WG # 4508, #5501, #5503 and #5504). We detected the highest mean signal intensity at WG #5504 of GPT-2 in overtly diabetic BBdp rats. The data suggest that GPT-2 isoforms have different immunoreactive epitopes. Two-dimensional Western blot of WG proteins probed with pooled sera from Finnish children newly diagnosed with diabetes also showed IgG binding to WG #5406 protein, while HLA-DQ matched non-diabetic children did not show any reactivity to these proteins (Figure 16).

Study #6: Homology of WG proteins to diabetes related proteins

The proteins identified by MS/MS analysis in Study #5 were compared to protein in the NCBI database to determine whether they shared structural homology with diabetes-related antigens using a pairwise Blast alignment of the respective amino acid sequences [79]. We scrutinised the results based on the length of the amino acid sequence that was identical and on the number of similar (charge) amino acids. Structural homologies between the immunogenic wheat peptides and diabetes related proteins are summarised in Table 7.

Table 7a: Barley GPT-2 homology to proteins related to diabetes (glutamate pyruvate transaminase- human)

Protein	Homology to diabetes-related antigens
Alanine amino-transferase 2 GPT-2 P52894	**glutamate pyruvate transaminase [Homo sapiens]** AAC51155 Blast search results: Dec 12 2001 Identities = 226/486 (46%), Positives = 316/486 (64%) Query: 2 AATVAVDNLNPKVLKCEYAVRGEIVIHAQRLQEQLKTQPGSLPFDEILYCNIGNPQSLGQ 61 A + +D +NP+V + EYAVRG IV A L+++L+ Q PF E++ NIG+ Q++GQ Sbjct: 18 AKVLTLDGMNPRVRRVEYAVRGPIVQRALELEQELR-QGVKKPFTEVIRANIGDAQAMGQ 76 Query: 62 QPVTFFREVLALCDHPDLLQREEIKTLFSADSISRAKQILAMIPGRATGAYSHSQGIKGL 121 +P+TF R+VLALC +PDLL F D+ RA++IL G + GAYS S GI+ + Sbjct: 77 RPITFLRQVLALCVNPDLLSSPN----FPDDAKKRAERILQACGGHSLGAYSVSSGIQLI 132 Query: 122 RDAIASGIASRDG-FPANADDIFLTDGASPGVHLMMQLLIRNE---KDGILVPIPQYPLY 177 R+ +A I RDG PA+ +++FL+ GAS + +++LL+ E + G+L+PIPQYPLY Sbjct: 133 REDVARYIERRDGGIPADPNNVFLSTGASDAIVTVLKLLVAGEGHTRTGVLIPIPQYPLY 192 (9) Query: 178 SASIALHGGALVPYYLNESTGWGLETSDVKKQLEDARSRGINVRALIINPGNPTGQV 237 SA++A G V YYL+E W L+ +++ + L AR RAL VINPGNPTGQV Sbjct: 193 SATLAELGAVQVDYYLDEERAWALDVAELHRALGQARDH-CRPRALCVINPGNPTGQVQT 251 Query: 238 EENQYDIVKFCKNEGLVLLADEVYQENIYVDNKKFHSFKKIVRSLG---YGEEDLPLVSY 294 E +++F E L LLADEVYQ+N+Y +FHSFKK++ +G G+++L S+ Sbjct: 252 RECIEAVIRFAFEERLFLLADEVYQDNVYAAGSQFHSFKKVLMEMGPPYAGQQEL--ASF 309 Query: 295 QSVSKGYYGECGKRGGYFEITGFSAPVREQIYKIASVNLCSNITGQILASLVMNPPKASD 354 S SKGY GECG RGGY E+ A V++Q+ K+ SV LC + GQ L LV++PP +D Sbjct: 310 HSTSKGYMGECGFRGGYVEVVNMDAAVQQQMLKLMSVRLCPPVPGQALLDLVVSPPAPTD 369 Query: 355 ESYASYKAEKDGILASLARRAKALEHAFNKLEGITCNEAEGAMYVFPQICLPQKAIEAAK 414 S+A ++AEK +LA LA +AK E FN+ GI+CN +GAMY FP++ LP +A+E A+ Sbjct: 370 PSFAQFQAEKQAVLAELAAKAKLTEQVFNEAPGISCNPVQGAMYSFPRVQLPPRAVERAQ 429 Query: 415 AANKAPDAFYALRLLESTGIVVVPGSGFGQVPGTWHFRCTILPQEDKIPAVISRFTVFHE 474 APD F+ LRLLE TGI VVPGSGFGQ GT+HFR TILP +K+ ++ + + FH Sbjct: 430 ELGLAPDMFFCLRLLEETGICVVPGSGFGQREGTYHFRMTILPPLEKLRLLLEKLSRFHA 489

Table 7b: Barley GPT-2 homology to proteins related to diabetes (glutamate pyruvate transaminase - Rat)

Alanine amino-transferase 2
GPT-2
P52894

glutamic-pyruvate transaminase (alanine aminotransferase) [Rattus norvegicus]
NP_112301
Blast search results: Dec 12 2001

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Identities = 224/482 (46%), Positives = 314/482 (64%),
Query: 5   VAVDNLNPKVLKCEYAVRGEIVIHAQRLQEQLKTQPGSLPFDEILYCNIGNPQSLGQQPV 64
           + +D +NP V + EYAVRG IV  A  L+++L+ Q    PF E++  NIG+ Q++GQ+P+
Sbjct: 21  LTLDTMNPCVRRVEYAVRGPIVQRALELEQELR-QGVKKPFTEVIRANIGDAQAMGQRPI 79

Query: 65  TFFREVLALCDHPDLLQREEIKTLFSADSISRAKQILAMIPGRATGAYSHSQGIKGLRDA 124
           TFFR+VLALC +P+LL   +    F  D+  RA++IL    G + GAYS S GI+ +R+
Sbjct: 80  TFFRQVLALCVYPNLLSSPD----FPEDAKRRAERILQACGGHSLGAYSISSGIQPIRED 135

Query: 125 IASGIASRDG-FPANADDIFLTDGASPGVHLMMQLLIRNE---KDGILVPIPQYPLYSAS 180
           +A  I  RDG  PA+ ++IFL+ GAS  +  M++LL+  E   + G+L+PIPQYPLYSA+
Sbjct: 136 VAQYIERRDGGIPADPNNIFLSTGASDAIVTMLKLLVSGEGRARTGVLIPIPQYPLYSAA 195

                                                          (9)
Query: 181 IALHGGALVPYYLNESTGWGLETSDVKKQLEDARSRGINVRALVVINPGNPTGQVLAEEN 240
           +A       V YYL+E   W L+ +++++ L  AR R    R L VINPGNPTGQV   E
Sbjct: 196 LAELDAVQVDYYLDEERAWALDIAELRRALCQARDR-CCPRVLCVINPGNPTGQVQTREC 254

Query: 241 QYDIVKFCKNEGLVLLADEVYQENIYVDNKKFHSFKKIVRSLG--YGEEDLPLVSYQSVS 298
              +++F   EGL L+ADEVYQ+N+Y +  +FHSFKK++  +G  Y  +   L S+ SVS
Sbjct: 255 IEAVIRFAFKEGLFLMADEVYQDNVYAEGSQFHSFKKVLMEMGPPYSTQQ-ELASFHSVS 313

Query: 299 KGYYGECGKRGGYFEITGFSAPVREQIYKIASVNLCSNITGQILASLVMNPPKASDESYA 358
           KGY GECG RGGY E+    A V++Q+ K+ SV LC  + GQ L  +V++PP  S+ S+
Sbjct: 314 KGYMGECGFRGGYVEVVNMDAEVQKQMGKLMSVRLCPPVPGQALMDMVVSPPTPSEPSFK 373

Query: 359 SYKAEKDGILASLARRAKALEHAFNKLEGITCNEAEGAMYVFPQICLPQKAIEAAKAANK 418
            ++AE+  +LA LA +AK  E  FN+  GI CN  +GAMY FPQ+ LP KA++ A+
Sbjct: 374 QFQAERQEVLAELAAKAKLTEQVFNEAPGIRCNPVQGAMYSFPQVQLPLKAVQRAQELGL 433

Query: 419 APDAFYALRLLESTGIVVVPGSGFGQVPGTWHFRCTILPQEDKIPAVISRFTVFHEAFMS 478
           APD F+ L LLE TGI VVPGSGFGQ  GT+HFR TILP  +K+  ++ + + FH  F
Sbjct: 434 APDMFFCLCLLEETGICVVPGSGFGQQEGTYHFRMTILPPMEKLRLLLEKLSHFHAKFTH 493
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Table 7c: Barley GPT-2 homology to wheat glutamate pyruvate transaminase

Alanine amino-transferase 2 (barley GPT-2) P52894	**alanine aminotransferase 2 transaminase 2 – (wheat GPT-2) The TIGR *Wheat* Gene Index (TaGI)** wheat|TC8197 Identities = 446/482 (92%), Positives = 459/482 (94%) Query: 1 MAATVAVDNLNPKVLKCEYAVRGEIVIHAQRLQEQLKTQPGSLPFDEILYCNIGNPQSLG 60 MAATVAVDNLNPKVLKCEYAVRGEIVIHAQRLQ+QL+TQPGSLPFDEILYCNIGNPQSLG Sbjct: 1637 MAATVAVDNLNPKVLKCEYAVRGEIVIHAQRLQQQLQTQPGSLPFDEILYCNIGNPQSLG 1458 Query: 61 QQPVTFFREVLALCDHPDLLQREEIKTLFSADSISRAKQILAMIPGRATGAYSHSQGIKG 120 QQPVTFFREVLALCDHPDLL++EEIKTLFSADSISRAKQILA+IPGRATGAYSHSQGIKG Sbjct: 1457 QQPVTFFREVLALCDHPDLLKKEEIKTLFSADSISRAKQILALIPGRATGAYSHSQGIKG 1278 Query: 121 LRDAIASGIASRDGFPANADDIFLTDGASPGVHLMMQLLIRNEKDGILVPIPQYPLYSAS 180 LRDAIASGIASRDGFPANADDIFLTDGASPGVHLMMQLLIRNEKDGILVPIPQYPLYSAS Sbjct: 1277 LRDAIASGIASRDGFPANADDIFLTDGASPGVHLMMQLLIRNEKDGILVPIPQYPLYSAS 1098 (9) Query: 181 IALHGGALVPYYLNESTGWGLETSDVKKQLEDARSRGINVRALVVINPGNPTGQVLAEEN 240 IALHGG+LVPYYLNESTGWGLE SDVKKQLEDARSRGI+VRALVVINPGNPTGQVLAEEN Sbjct: 1097 IALHGGSLVPYYLNESTGWGLEISDVKKQLEDARSRGIDVRALVVINPGNPTGQVLAEEN 918 Query: 241 QYDIVKFCKNEGLVLLADEVYQENIYVDNKKFHSFKKIVRSLGYGEEDLPLVSYQSVSKG 300 QYDIVKFCKNEGLVLLADEVYQENIY DNKKFHSFKKIVRSLGYGEEDLPLVSYQSVSKG Sbjct: 917 QYDIVKFCKNEGLVLLADEVYQENIYADNKKFHSFKKIVRSLGYGEEDLPLVSYQSVSKG 738 Query: 301 YYGECGKRGGYFEITGFSAPVREQIYKIASVNLCSNITGQILASLVMNPPKASDESYASY 360 YYGECGKRGGY EITGFSAPVREQIYKIASVNLCSNITGQILASLVMNPPKASDESYASY Sbjct: 737 YYGECGKRGGYMEITGFSAPVREQIYKIASVNLCSNITGQILASLVMNPPKASDESYASY 558 Query: 361 KAEKDGILASLARRAKALEHAFNKLEGITCNEAEGAMYVFPQICLPQXXXXXXXXXXXXX 420 KAEKDGILASLARRAKALE AF+KLEGITCN+AEGAMY+FPQICLPQ Sbjct: 557 KAEKDGILASLARRAKALEVAFSKLEGITCNKAEGAMYLFPQICLPQKAIEAAKAANKAP 378 Query: 421 XXXXXLRLLESTGIVVVPGSGFGQVPGTWHFRCTILPQEDKIPAVISRFTVFHEAFMSEY 480 LRLLESTGIVVVPGSGFGQVPGTWHFRCTILPQE+KIPA+ISRFTVFHE FM+EY Sbjct: 377 DAFYALRLLESTGIVVVPGSGFGQVPGTWHFRCTILPQEEKIPAIISRFTVFHETFMAEY 198 Query: 481 RD 482 RD Sbjct: 197 RD 192

Table 7d: Barley GPT-2 homology to wheat ornithine aminotransferase and proteins related to diabetes (HSP60 -human)

Alanine amino-transferase 2 GPT-2 P52894	**ornithine/acetylornithine aminotransferase [Triticum aestivum].** AAB80947 Blast search results: Dec 13 2001 Identities = 10/33 (30%), Positives = 15/33 (45%) Query: 89 FSADSISRAKQILAMIPGRATGAYSHSQGIKGL 121 F +++I IL + PG AY + GL Sbjct: 97 FISEAIQGVGGILELAPGYLPAAYDMVRKAGGL 129
	60 KDA HEAT SHOCK PROTEIN, MITOCHONDRIAL PRECURSOR (HSP60) (60 KDA CHAPERONIN) (CPN60) (HEAT SHOCK PROTEIN 60) (human) 129379 Blast 2 sequence results: Dec 12 2001 Identities = 13/61 (21%), Positives = 26/61 (42%), Query: 83 EEIKTLFSADSISRAKQILAMIPGRATG-AYSHSQGIKGLRDAIASGIASRDGFPANADD 141 E + TL ++R K L ++ +A G + +K + A + +G N +D Sbjct: 281 EALSTLV----LNRLKVGLQVVAVKAPGFGDNRKNQLKDMAIATGGAVFGEEGLTLNLED 336
	DiaPep277 peptide of HSP60 (VLGGGVALLRVIPALDSLTPANED) (human) Blast 2 sequences results: Nov 29 2001 Identities = 4/7 (57%), Positives = 6/7 (85%) Query: 6 AVDNLNP 12 A+D+L P Sbjct: 14 ALDSLTP 20

Table 7e: Wheat GPT-2 and barley GB-1 homology to proteins related to diabetes (HSP60 –human, DiaPep277)

aminotransferase 2 transaminase 2 (wheat GPT-2) wheat|TC8197	**60 KDA HEAT SHOCK PROTEIN, MITOCHONDRIAL PRECURSOR (HSP60) (60 KDA CHAPERONIN) (CPN60) (HEAT SHOCK PROTEIN 60) (human)** 129379 Blast 2 sequence results: Dec 14 2001 Identities = 29/112 (25%), Positives = 47/112 (41%) Query: 222 GYISPYFINTSKGQKCEFQDAYVLLSEKKISSIQSIVPALEIANAHRKPLVIIAEDVDGE 281 G + PY++N S G E D L + + I V AL + N ++AE+ + Sbjct: 276 GSLVPYYLNESTGWGLEISDVKKQLEDARSRGID--VRALVVINPGNPTGQVLAEENQYD 333 Query: 282 ALSTLVLNRLKVGLQVVAVKAPGFGDNRK-NQLKDMAIATGGAVFGEEGLTL 332 + N V L + + DN+K + K + + G +GEE L L Sbjct: 334 IVK-FCKNEGLVLLADEVYQENIYADNKKFHSFKKIVRSLG---YGEEDLPL 381
	DiaPep277 peptide of HSP60 (VLGGGVALLRVIPALDSLTPANED) (human) Blast 2 sequences results: Dec 14 2001 Identities = 4/7 (57%), Positives = 6/7 (85%) Query: 6 AVDNLNP 12 A+D+L P Sbjct: 14 ALDSLTP 20
globulin Beg1 precursor GB-1 S35221	**60 KDA HEAT SHOCK PROTEIN, MITOCHONDRIAL PRECURSOR (HSP60) (60 KDA CHAPERONIN) (CPN60) (HEAT SHOCK PROTEIN 60) (human)** 129379 Blast 2 sequence results: Dec 12 2001 Identities = 10/30 (33%), Positives = 16/30 (53%), Query: 275 PAGSIMHLANTDGRRKLVIAKILHTISVPG 304 PA +I A +G L++ KI+ + S G Sbjct: 475 PAMTIAKNAGVEG--SLIVEKIMQSSSEVG 502

Table 7f: Barely GB-1 homology to proteins related to diabetes (DiaPep277, 11S glycinin, insulin receptor-human)

globulin Beg1 precursor GB-1 S35221	**DiaPep277 peptide of HSP60 (VLGGGVALLRVIPALDSLTPANED)** (human) Blast 2 sequence results: Nov 29 2001 Identities = 7/16 (43%), Positives = 8/16 (49%) Query: 304 GKFQFLSVKPLLASLS 319 G L V P L SL+ Sbjct: 4 GGVALLRVIPALDSLT 19
	11S Glycinin (Glycinin G1 Precursor) – soybean P04776 Blast 2 sequences results: Dec 28 2001 Identities = 10/31 (32%), Positives = 18/31 (57%) Query: 217 IRDYRVAIMEVNPRAFVVPGFTDADGVGYVA 247 +++ RV I+ P+ FVV + +D YV+ Sbjct: 406 LQEGRVLIV---PQNFVVAARSQSDNFEYVS 433
	Insulin receptor precursor - human INHUR Blast 2 sequence results: Dec 12 2001 Identities = 21/70 (30%), Positives = 34/70 (48%), Query:213 LLRGIRDYRVAIMEVNP----------RAFVVPGFTDADGV---GYVAQGEGVLTVIE 257 +++G + RVA+ VN A V+ GFT V G V++G+ L V+E Sbjct:1045 IIKGEAETRVAVKTVNESASLRERIEFLNEASVMKGFTCHHVVRLLGVVSKGQPTLVVME 1104

Table 7g: Barley GB-1 and wheat WSG homology to proteins related to diabetes (IL-1 beta- rat, IL-1 receptor- rat, HSP 60)

globulin Beg1 precursor GB-1 S35221	**interleukin 1 beta [Rattus norvegicus]** NP_113700 Blast 2 sequence results: Dec 12 2001 Identities = 6/20 (30%), Positives = 11/20 (55%) Query:379 PPFRGDSRDTFNLLEQRPKI 398 P + + R FN +E + K+ Sbjct:207 PKKKMEKRFVFNKIEVKTKV 226
	interleukin 1 receptor, type I [Rattus norvegicus] NP_037255 Blast 2 sequence results: Nov 12 2001 Identities = 6/19 (31%), Positives = 11/19 (57%) Query: 181 RPYVFGPRSFRRIIQSDHG 199 RP + PR+ +++D G Sbjct: 225 RPVIMSPRN--ETMEADPG 241
Wheat storage globulin (WSG) AAA34269	**60 KDA HEAT SHOCK PROTEIN, MITOCHONDRIAL PRECURSOR (HSP60) (60 KDA CHAPERONIN) (CPN60) (HEAT SHOCK PROTEIN 60) (human)** 129379 Blast 2 sequence results: Dec 28 2001 Identities = 10/30 (33%), Positives = 16/30 (53%) Query: 275 PAGSIMHLANTDGRRKLVIAKILHTISVPG 304 PA +I A +G L++ KI+ + S G Sbjct: 475 PAMTIAKNAGVEG--SLIVEKIMQSSSEVG 502

Table 7h: WSG homology to proteins related to diabetes (HSP60 –human, 11S glycinin, insulin receptor-human)

Wheat storage globulin (WSG) AAA34269	**DiaPep277 peptide of HSP60 (VLGGGVALLRVIPALDSLTPANED)** (human) Blast 2 sequence results: Dec 28 2001 Identities = 7/16 (43%), Positives = 8/16 (49%) Query: 304 GKFQFLSVKPLLASLS 319 G L V P L SL+ Sbjct: 4 GGVALLRVIPALDSLT 19
	11S Glycinin (Glycinin G1 Precursor) – soybean P04776 Blast 2 sequences results: Dec 28 2001 Identities = 10/31 (32%), Positives = 18/31 (57%) Query: 217 IRDYR[redacted]RAFVVPGFTDADGVGYVA 247 +++ R[redacted] FVV + +D YV+ Sbjct: 406 LQEGRVLIV---PQNFVVAARSQSDNFEYVS 433
	Insulin receptor precursor - human INHUR Blast 2 sequence results: Dec 12 2001 Identities = 21/70 (30%), Positives = 34/70 (48%), Query:213 LLRGIRDYRV[redacted]NP----------RAFVVPGFTDADGV---GYVAQGEGVLTVIE 257 +++G + RV[redacted] A V+ GFT V G V++G+ L V+E Sbjct:1045 IIKGEAETRVAVKTVNESASLRERIEFLNEASVMKGFTCHHVVRLLGVVSKGQPTLVVME 1104

Table 7i: WSG homology to proteins related to diabetes (IL-1 beta - rat, IL-1 receptor - rat)

Wheat storage globulin (WSG) AAA34269	**interleukin 1 beta [Rattus norvegicus]** NP_113700 Blast 2 sequence results: Dec 28 2001 Identities = 12/56 (21%), Positives = 23/56 (40%) Query: 538 NLQVVCFEINAERNERVWLAGRNNVIGK------LGSPAQELTFGRPAREVQEVFR 587 N ++ F+ +E N+ + A R I LG P + + + + + FR Sbjct: 8 NCEIAAFD--SEENDLFFEADRPQKIKDCFQALDLGCPDESIQLQISQQHLDKSFR 61
	interleukin 1 receptor, type I [Rattus norvegicus] NP_037255 Blast 2 sequence results: Dec 28 2001 Identities = 11/25 (44%), Positives = 16/25 (64%) Query: 439 NTQ-SFKLAVVLEGEGEVQIVCPHL 462 NTQ SF + + G+G +VCP+L Sbjct: 123 NTQASFIQRLHVAGDGS--LVCPYL 145

GPT-2 from barley (Acc. No. P52894) had 46% (226/486) identical amino acid sequence and 64% (316/486) of the amino acids had the same charge as glutamate pyruvate transaminase in humans (Acc. No. AAC51155, Table 7a). Similar homologies were found in the rat glutamate pyruvate transaminase (Acc. No. NP_112301, Table 7b). Wheat alanine aminotransferase 2 transaminase 2 (GPT-2, TC# 8197) is 92% (446/482) identical and 94% similar in charge (49/482) to barley GPT-2 (Table 7c). One of the peptide fragments we detected in our MS/MS analysis of WG #5503 (peptide #9) is found in the GPT-2 sequence from all three sources of protein: human, rat and wheat.

Barley GPT-2 (Acc. No. P52894) had 13/61 (21%) amino acid identity and 26/61 (42%) amino acids of the same charge as heat shock protein 60 isolated from humans (Acc. No. 129379, Table 7d). HSP60 has been proposed as a possible autoantigen in T1D [129, 130] or as a regulator of the progression of insulitis because it stimulates IL-10 production [131]. Next, we aligned the barley GPT-2 (barley) with an immunogenic peptide of HSP60 (Diapep277). We found 4/7 (57%) of the amino acids were identical and 6/7 (85%) amino acids were of similar charge as DiaPep277 (Table 7d). We repeated these homology comparisons with wheat GPT-2 (TC# 8197, Table 7e). Wheat GPT-2 was 25% (29/112) identical in its amino acid sequence and 41% (47/112) of the amino acids showed similar charge as those in human HSP60 (Acc. No. 129379). Moreover, the immunogenic peptide DiaPep277 was 30% identical (10/30) and 85% similar in charge (6/7) to wheat GPT-2 (Table 7e).

We queried the amino acid sequence of globulin Beg1 precursor (GB-1, barley) in the NCBI database to find structurally homologous diabetes-related proteins. Barley GB-1 (Acc. No. S35221) had 10/30 (33%) amino acids that were identical and 16/30 (53%)

amino acids of the same charge as human HSP60 (Acc. No. 1293701, Figure 7e). The immunogenic Diapep277 of HSP60 was also identical in 7/16 (43%) amino acids to barley GB-1 (Acc. No. S35221, Table 7f). Another diabetes related homologous protein of interest was the insulin receptor. We aligned the sequence of barley GB-1 (Acc. No. S35221) with that of the human insulin receptor precursor (Acc. No. INHUR), and found 30% (21/70) amino acids were identical and 48% (34/70) amino acids were of the same charge (Table 7f). Sequence alignment of the rat interleukin-1 (IL-1) receptor (Acc. No. NP_037255) with GB-1 (Acc. No. S35221) showed that 6/19 (31%) amino acids were identical and 11/19 (57%) were of similar charge (Table 7g). The IL-1 cytokine (rat) was homologous in 6/20 (30%) amino acids to barley GB-1 (Acc. No. S35221). Subsequently, we repeated the homology queries with WSG (Acc. No. AAA34269) and found the same homologous sequences as with GB-1 (Table 7g, 7h and 7i). This is not surprising given that the sequences of GB-1 and WSG are identical (Table 6e and 6f).

These structurally homologous sequences may be recognised by autoreactive T cells. If dietary antigens activate these autoreactive T cells, the immune response could spread to homologous self proteins and initiate an autoimmune reaction. The biological significance of these sequence similarities needs to be tested *in vitro* to show functional molecular mimicry.

Study #7: 1D WG Western blots with serum from Finnish children newly diagnosed with diabetes

In Study #3, we showed differences in the antigenicity of various WG proteins using BB rat sera in semi-quantitative Western blot analysis. We applied the same methodology to study antibody binding to WG proteins in Finnish children with diabetes and non-diabetic HLA-matched control children.

A Western blot of WG proteins was probed with cross-sectional serum samples and the level of IgG binding in cases and controls was compared by densitometry. We screened 23 patients with diabetes and 41 control children from Finland (Table 8). Four non-diabetic control children were outliers because individual wheat protein bands could not be identified because the intensity of the signal was too high (Appendix I, page 143) and they were excluded from the analysis. One-dimensional Western blots of WG proteins probed with sera from Finnish children are shown in Figure 16. A plot of the mean signal intensity detected on the Western blots suggested that newly diabetic children had increased reactivity to 25, 33, 92 and 105 kDa wheat gluten proteins compared with non-diabetic controls (p=0.03-0.005, Figure 17). However, the frequency of children with IgG antibodies to wheat gluten proteins was similar in both risk groups, except in the case of the 33 kDa proteins (Figure 18). Eighty–three percent (19/23) of newly diagnosed children with diabetes had IgG antibodies to 33 kDa WG proteins, while 57% (21/37) of control children had detectable (>0.1 OD) IgG binding (p=0.04).

The caveat in comparing pooled sera from children with and without diabetes is that some individuals, depending on their HLA haplotypes, have stronger humoral and cellular immunity to various antigens. For instance the HLA A1-B8-DR3-DQ2 haplotype was associated with increased immune responses to GAD [132]. To minimize the influence of this confounding factor in our Western blot analysis, we performed a pairwise comparison of the IgG reactivity to WG in newly diabetic children and non-diabetic control children matched for age, sex and HLA DQ genotype (n=23 pairs). We evaluated various combinations of WG protein bands detected in the Western blot to find the pattern of antigenic bands that was associated with diabetes.

Table 8: Description of newly diagnosed patients with type 1 diabetes and non-diabetic (control) children from Finland

Patient number	Group	Sex	Age	Patient number	Group	Sex	Age
2A	control	F	3.2	2	diabetic	F	3.3
2B	control	F	3.3	3	diabetic	M	3.4
3B	control	M	3.5	4	diabetic	F	4.3
4B	control	F	4.4	5	diabetic	F	5.5
5B	control	F	6.1	6	diabetic	F	6.5
6A	control	F	6.5	7	diabetic	M	7.8
6B	control	F	6.3	11	diabetic	F	8.8
7A	control	M	8.1	12	diabetic	F	8.9
11A	control	F	8.2	13	diabetic	F	9.5
12A	control	F	8.5	14	diabetic	M	9.7
12B	control	F	9.4	15	diabetic	F	10.0
13A	control	F	9.6	17	diabetic	F	10.2
13B	control	F	8.8	18	diabetic	F	10.3
14A	control	F	9.5	19	diabetic	M	10.6
14B	control	F	9.6	20	diabetic	F	10.7
15B	control	F	10.1	21	diabetic	M	11.1
17A	control	F	10.3	22	diabetic	F	11.8
17B	control	F	10.1	23	diabetic	F	12.5
18A	control	F	10.7	24	diabetic	M	13.1
18B	control	F	10.5	25	diabetic	F	13.6
19A	control	M	10.8	26	diabetic	F	14.1
20B	control	F	11.1	27	diabetic	F	14.6
21A	control	M	11.4	28	diabetic	M	15.1
21B	control	M	12.4				
22A	control	F	11.3				
22B	control	F	11.6				
23A	control	F	12.3				
23B	control	F	11.7				
24B	control	F	13.2				
25A	control	F	12.3				
25B	control	F	12.0				
26A	control	F	3.5				
26B	control	F	13.0				
27A	control	F	17.6				
27B	control	F	14.4				
28A	control	F	14.4				
28B	control	M	15.7				

Figure 17 Mean signal intensity detected in Western blot of wheat gluten proteins probed with sera from Finnish children newly diagnosed with diabetes and non-diabetic control children.

Western blot of WG proteins was probed with serum from 23 children newly diagnosed with diabetes and 37 non-diabetic control children. IgG binding was detected using anti-human IgG antibody labeled with HRP, which was visualized by enhanced chemiluminescence. Mean absorbance $\pm$ SD of each WG protein band is shown as a bar graph. ANOVA and post hoc comparison of means using LSD test of case and controls; § indicates p=0.02-0.03, ‡ indicated p=0.005.

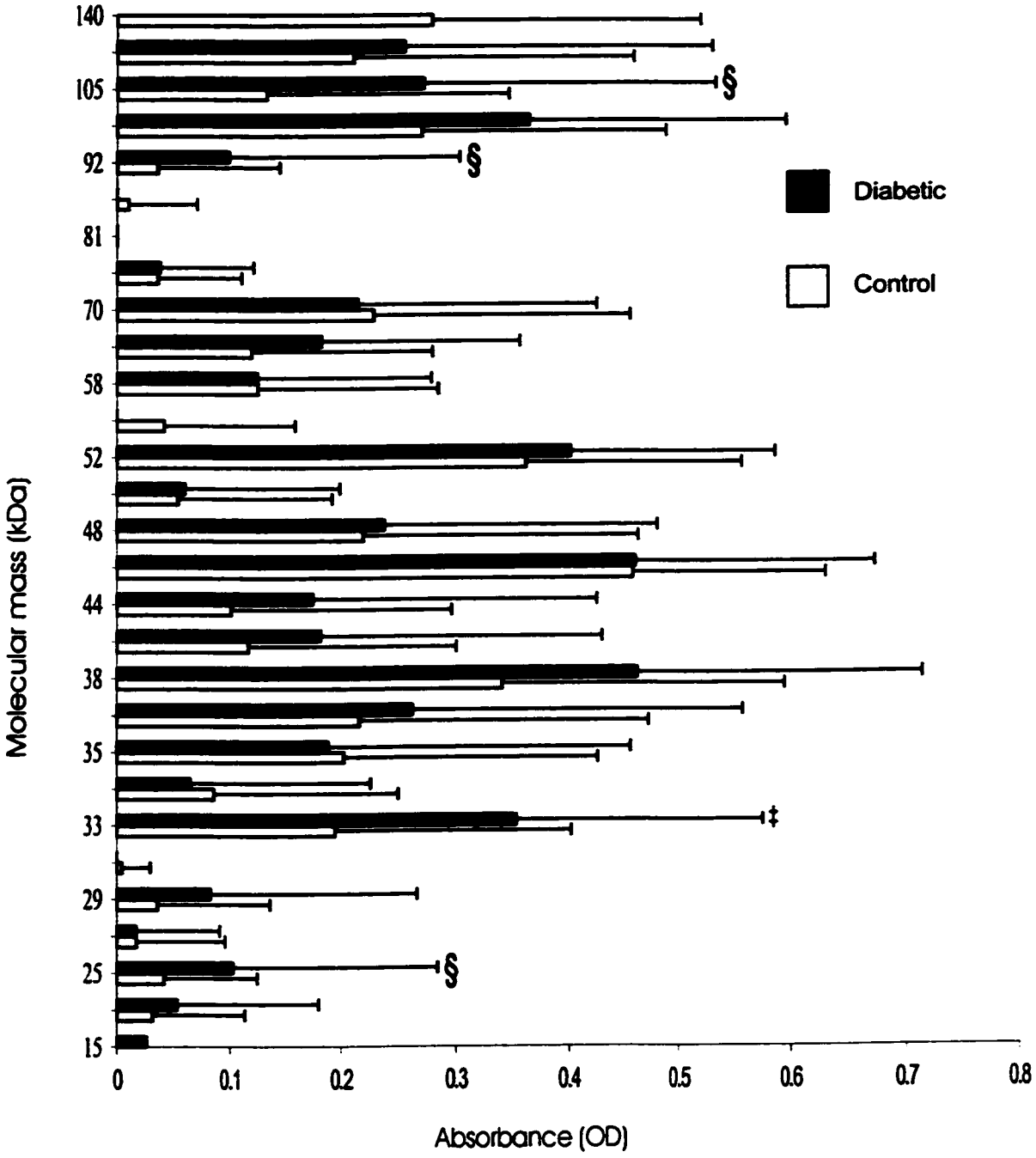
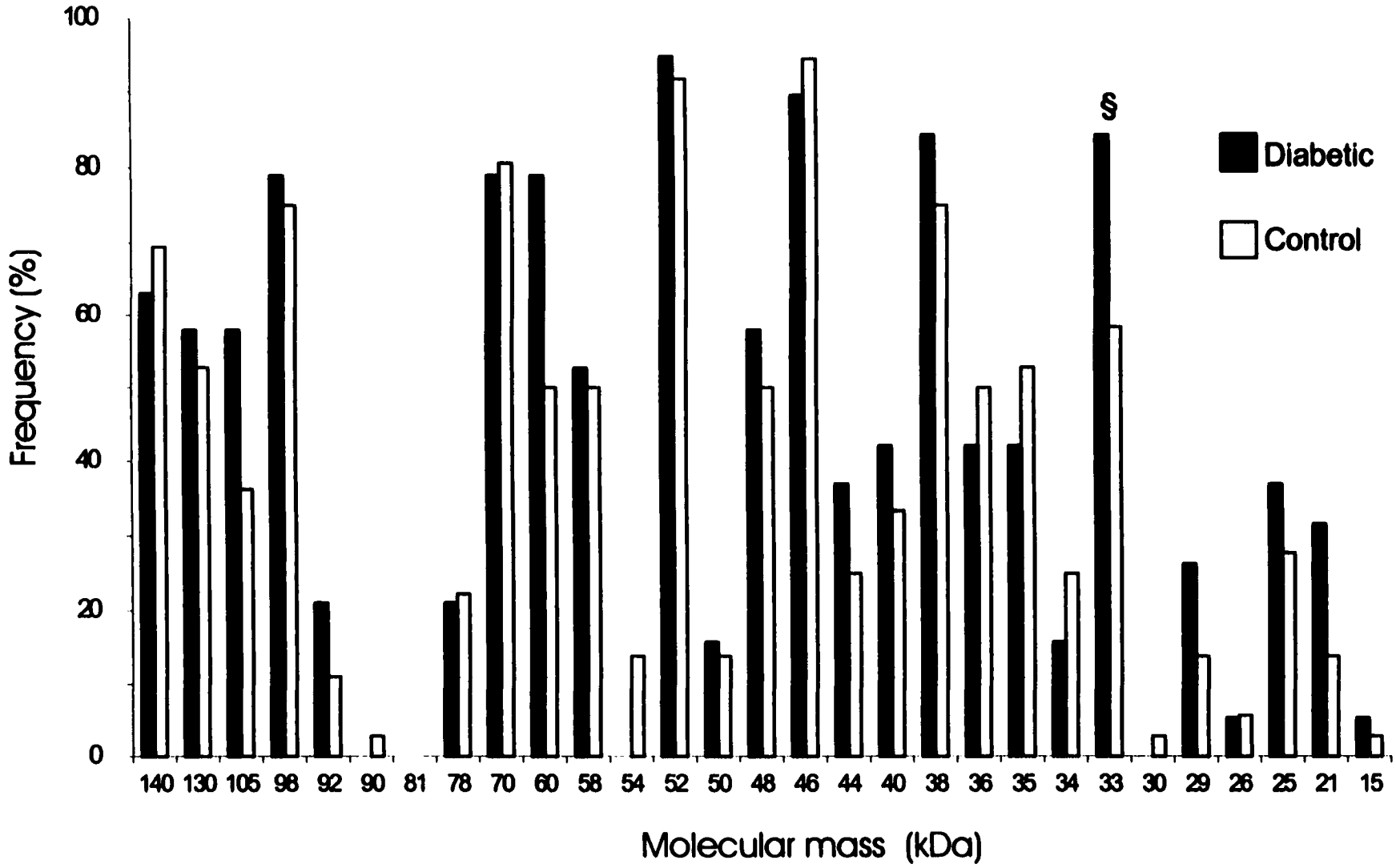

Molecular mass (kDa)
140
105
92
81
70
58
52
48
44
38
35
33
29
25
15
§
§
§
‡
Diabetic
Control
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
Absorbance (OD)

Figure 18 Frequency of Finnish children newly diagnosed with diabetes and non-diabetic control children with IgG antibodies to wheat gluten proteins

Western blot of WG proteins was probed with serum from 23 children newly diagnosed with diabetes and 37 non-diabetic control children. IgG binding was detected using anti-human IgG antibody labeled with HRP, which was visualized by enhanced chemiluminescence. Mean absorbance of each WG protein band is shown as abar graph. Fisher's Exact test was performed to compare frequency of cases and controls; § indicates p=0.04.


100
80
60
40
20
0
Frequency (%)
§
Diabetic
Control
140 130 105 98 92 90 81 78 70 60 58 54 52 50 48 46 44 40 38 36 35 34 33 30 29 26 25 21 15
Molecular mass (kDa)

We compared 23 case-control HLA-DQ-matched pairs to determine whether concomitant reactivity to combinations of WG proteins was associated with diabetes. The combination of 33 and 38 kDa proteins was detected in 74% (17/23) of children with diabetes from Finland, whereas it was found in 22% (5/23) HLA-matched non-diabetic controls (Table 9, p=0.0009). Higher signal intensity was detected in 19/23 (83%) diabetic case-control pairs to 33 kDa proteins (Figure 19). Moreover, higher antibody binding was detected in 17 children with diabetes of the 23 case-control pairs (74%) (Figure 19).

Furthermore, a Receiver Operating Characteristic (ROC) plot (Figure 20) was constructed to test the sensitivity and specificity of the 33 and 38 kDa band pattern as a surrogate marker of diabetes. Various cutoff OD values (Table 10) were evaluated to find the one with the highest discriminating ability (largest distance from non-informative curve). In the diabetic patients, the concomitant presence of the 33 kDa and 38 kDa bands, when the 33 kDa band has an OD value of at least 0.32 or the 38 kDa value has an OD of at least 0.58 (believe-the-positive rule), had the highest specificity (87%) and sensitivity (78%).

Figure 19 Pairwise comparison of absorbance detected at wheat gluten protein bands in children newly diagnosed with diabetes and the genetically matched non-diabetic control children from Finland

Children newly diagnosed with diabetes were HLA-matched with a non-diabetic control child (n= 23 case-control pairs). Western blot of WG proteins was probed with sera from 23 case-control pairs to detect level of IgG binding to WG proteins.

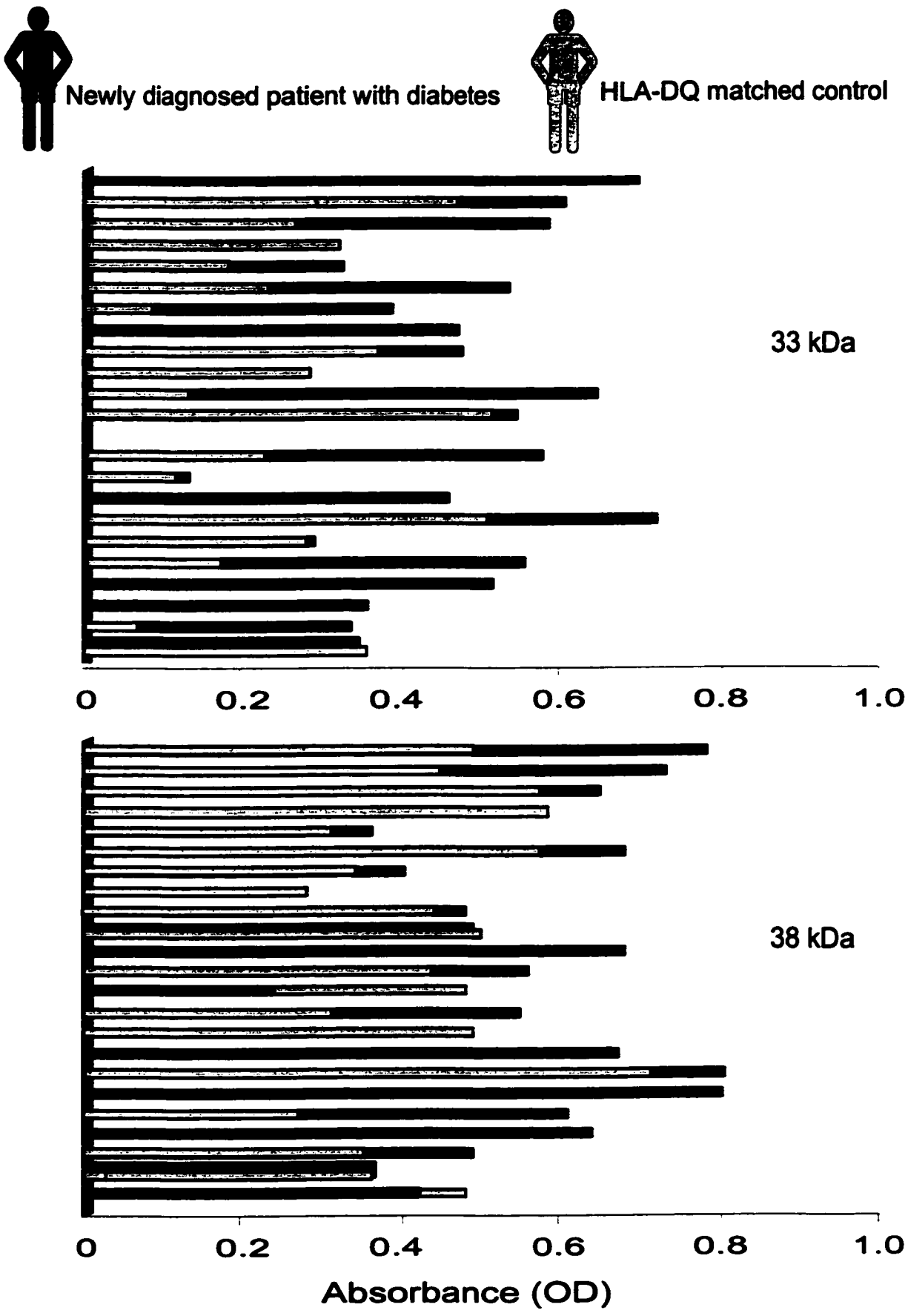

Newly diagnosed patient with diabetes
HLA-DQ matched control
33 kDa
0
0.2
0.4
0.6
0.8
1.0
38 kDa
0
0.2
0.4
0.6
0.8
1.0
Absorbance (OD)

Figure 20 Receiver Operating Characteristic (ROC) Plot

Different absorbance cutoff values were evaluated for 33 and 38 kDa WG proteins to find the conditions with the highest specificity and sensitivity (highest discriminating ability).The data point at the largest distance (specificity 87% and specificity 78%) from the non-informative line is the best surrogate marker (detection of 33 +38 kDa IgG antibodies, when absorbance at 38 kDa protein $\geq$ 0.58 OD or at 33 kDa $\geq$ 0.32 OD).

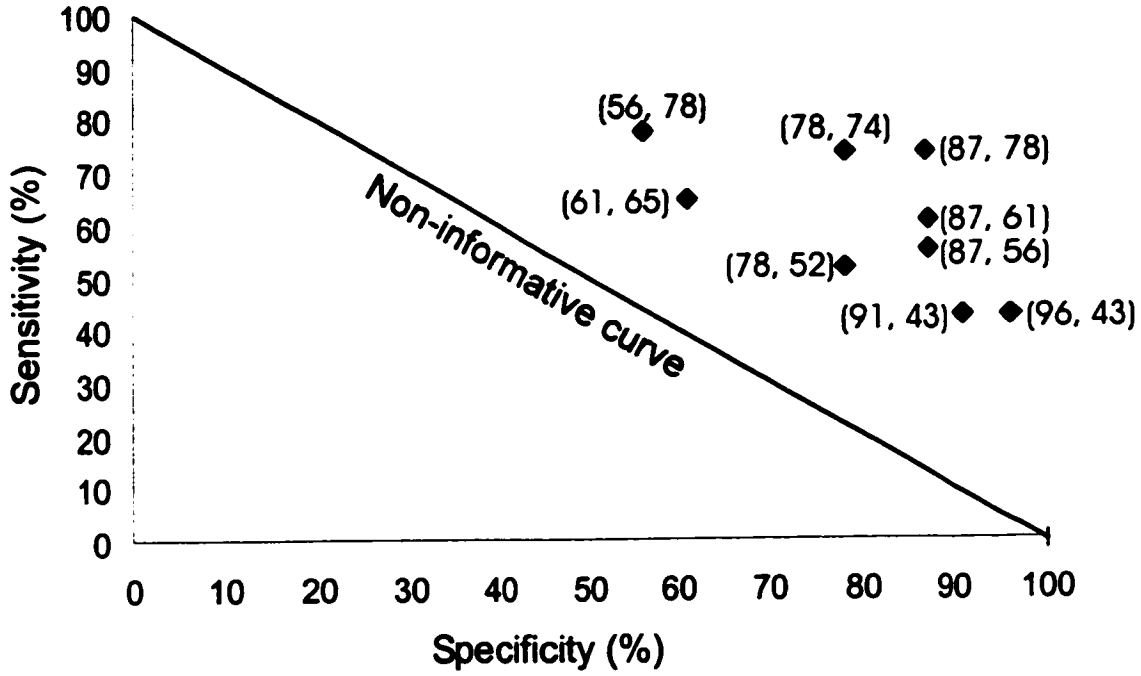

100
90
80
70
60
50
40
30
20
10
0
Sensitivity (%)
0
10
20
30
40
50
60
70
80
90
100
Specificity (%)
Non-informative curve
(56, 78)
(78, 74)
(87, 78)
(61, 65)
(87, 61)
(87, 56)
(78, 52)
(91, 43)
(96, 43)

Table 9: Frequency of HLA-DQ matched case-control pairs of children from Finland with IgG antibodies to combinations of wheat gluten proteins

WG proteins (kDa)	Number of case-control pairs (n=23) with IgG antibodies to WG proteins		
	T1D % (n)	Control % (n)	p-value
33+38+98+105	39 (9)	4 (1)	0.01
33+38+105	39 (9)	0 (0)	0.001
33+38	74 (17)	22 (5)	0.0009
33+98	56 (13)	4 (1)	0.0001
98+105	43 (10)	13 (3)	0.02
33+105	43 (10)	0 (0)	0.00003
33+46	70 (16)	7 (2)	0.000001
38+46	70 (16)	4 (1)	0.0000001
38+52	56 (13)	13 (3)	0.002
33+38+98	52 (12)	7 (2)	0.003

Table 10: Evaluation of the specificity and sensitivity of IgG reactivity to 33 and 38 kDa wheat gluten proteins on Western blots probed with sera from Finnish children

Wheat proteins (kDa)	Absorbance Cutoff (OD)	Specificity (%)	Sensitivity (%)
38	0.58	91	43
38	0.50	78	52
38	0.44	61	65
38	0.36	56	78
33	0.48	96	43
33	0.36	87	55
33	0.32	78	74
33 + 38	0.58 (38 kDa) or 0.32(33kDa)	87	78
33 + 38	0.58(38kDa) or 0.36(33kDa)	87	61

DISCUSSION

There is substantial evidence that T1D in BBdp rats and NOD mice is food induced. Wheat is one of the major diabetes-promoting food sources but the identity of wheat antigens has not been reported. We characterised the amino acid sequence of antigenic wheat proteins that may be candidate antigens implicated in the development of T1D. Our results provide evidence of antigenic wheat proteins relevant to diabetes that are homologous with self-proteins, particularly proteins relevant to the autoimmune process.

Using antibodies to define diabetes-related wheat proteins

Our investigation used an antibody-based approach to characterise the antigenic wheat proteins that promote development of T1D. Most of the β-cell autoantigens identified so far have been characterised using similar studies of IgG antibody reactivity in sera of diabetic patients and rodents. For example, preliminary studies aimed at characterising the nature of the primary β-cell target antigens were based on antibodies in sera of BB rats that recognised rat islet cell proteins of 64-kDa [133]. Subsequent investigations of the humoral reactivity in sera from patients with Stiff-man syndrome and T1D have identified the 64-kDa autoantigen as one of the major autoantigens in diabetes, glutamic acid decarboxylase (GAD65) [84]. The identification of these autoantigens has facilitated investigations of the disease process. Mathematical models of the levels of circulating autoantibodies have been derived to estimate the time to diabetes onset [122].

Recently, the 1st International NOD Mouse T cell Workshop concluded that antibody-based assays (i.e. ELISPOT and ELISA analysis of cytokines) are preferred for

the study of autoreactive T cells because assays that measure T-cell proliferation in response to β-cell autoantigens are difficult to standardise [134]. Subtle technical aspects (preparation of T cells or choice of reagents) are associated with significant variations in T cell responses. Moreover, T cells of interest reside in the mesenteric or pancreatic lymph nodes or in the pancreas itself, which makes sample collection and prospective follow-up problematic in rodents and impossible in humans.

T1D has been proposed to be a T cell mediated disease because activated helper T cells (Th) have been detected in islet lesions of newly diagnosed patients [4]. The cytokine profile of these Th cells and the response they evoke has been used to classify them as type 1 (Th1, IL-2, IFN-γ, TNF-β, cell mediated immunity) and type 2 (Th2, IL-4, IL-5, IL-6, IL-10, humoral immunity) [135]. In insulitis, Th2 cells in the islet lesions interact with B-lymphocytes to promote humoral immune responses. B cells bind antigen by their surface immunoglobulin receptor, internalise the antigen and present it on MHC class II molecules to enlist the help of T cells. Th2 cells that recognise the antigen presented on the MHC class II molecules secrete cytokines (IL-4, IL-5, IL-6 and IL-10) that are required for B cell proliferation and differentiation into antibody secreting lymphocytes. Therefore, studies of antibody reactivity are also a reflection of T cell reactivity.

We have detected differences in IgG binding to WG proteins that were associated with susceptibility to diabetes. Our cross-sectional study showed that BBc rats had less IgG binding to WG proteins than BBdp animals (Figure 6). However, the timing of the development of diabetes differs from animal to animal, ranging from 50-140 d, and some BBdp rats never exhibit clinical diabetes. Therefore, animals were followed prospectively

to determine whether the increased antibody binding to WG proteins in BBdp rats was associated with the development of T1D. Both prospective studies (84F and 86F) of WG-fed BBdp rats showed that higher antibody reactivity to low molecular mass WG proteins (33, 36 and 38 kDa) was detected in pre-diabetic BB rats. Differences between asymptomatic and pre-diabetic rats in the level and frequency of reactivity to these LMM wheat proteins suggest that these proteins were processed or handled differently by the gut of pre-diabetic animals.

Our findings could be related to differences in the permeability of the intestine of BBdp and BBc rats [136]. When mannitol, a usually non-degradable and non-absorbed sugar alcohol that serves as an indicator of gut permeability was administered orally to BB rats, higher levels of mannitol were detected in the blood of BBdp than in BBc rats [137]. In addition, gastric and small intestinal permeability to sugars and sugar alcohols was increased in BBdp rats [136]. This suggests that the gut mucosa of BBdp rats may be more permeable to dietary antigens. Hence, the differences in IgG reactivity to WG may be associated with more WG proteins coming in contact with the GALT. Abnormalities in gut permeability are one of the hallmarks of gut inflammation.

There are several indications that the gut of BBdp rats is in a pro-inflammatory state following exposure to cereal-based diets such as NIH-07. For example, higher levels of myeoloperoxidase activity, a measure of neutrophil infiltration, were detected in the small intestine of 60-90 d old BBdp rats (non-diabetic) compared with BBc rats [138]. BBdp rats fed a cereal-based rodent chow exhibited increased permeability to normally excluded molecules [136] and increased IL-1β stimulated NO production [139]. Similarly, others have reported increased eosinophil infiltration in the jejunum of

suckling rats fed cow's milk, another potential diabetes promoting food, compared to those fed exclusively mother's milk [140]. Further evidence of gut inflammation in the BB rat was shown in a study of pro-inflammatory intracellular cytokines. A higher frequency of IFN-γ^+ $CD3^+CD4^+$ T cells from MLN and Peyer's patches was detected in 45 day old BBdp rats compared to BBc rats fed the cereal NIH-07 diet [141]. *In vitro* studies have shown that IFN-γ can cause the intestinal epithelial monolayer to become leaky [142], suggesting that a local pro-inflammatory response may increase the antigen load in the GALT. In contrast, the HC-fed BBdp rats showed a decrease in the prevalence of IFN-$\gamma^+CD3^+CD4^+$ T cells, suggesting that dietary antigens modulated immune activation in the GALT. However, the cause of the gut damage and whether immune activation in the GALT is a crucial requirement in the development of diabetes or is an outcome of the diabetes process are issues that remain to be resolved.

The differences in antibody reactivity in BBc and BBdp rats may also be a reflection of changes in immune regulation. BBdp animals are lymphopenic and so they lack regulatory T cells in the GALT that could modulate immune reactivity to dietary antigens. Whalen *et al.* characterised a population of lymph node regulatory T cells expressing the ART2 antigen (RT6) that were shown to prevent diabetes when transferred to BBdp rats [143]. By contrast, depletion of ART2 cells in diabetes-resistant (BBDR) rats induced T1D. Hence, BBdp rats may have at least two weaknesses that predispose them to T1D, first their gut is more permeable to antigens in the lumen, and second, they have a deficit of T cells that regulate immune responses to dietary antigens and control the outcome of immune responses.

We studied whether a similar trend in antibody reactivity to WG proteins would be detected in Finnish children newly diagnosed with T1D. Western blots showed that the same combination of 33- and 38-kDa wheat protein bands was more immunogenic in children with diabetes than in control children without diabetes (Table 9). We examined the sensitivity and specificity of a semi-quantitative analysis of the level of IgG binding on Western blots as a surrogate marker of T1D (Table 10). Detection of IgG binding to 33- and 38-kDa wheat proteins, when the absorbance at the 33 kDa WG proteins is at least 0.32 OD or the absorbance at the 38 kDa WG proteins is at least 0.58 OD, was associated with 87% specificity and 78% sensitivity. Therefore, increased antibody reactivity to 33 and 38 kDa wheat proteins was associated with T1D.

When the level of reactivity to WG proteins was compared in HLA-DQ-matched pairs of cases and controls, the children with diabetes had higher IgG binding to 33 and 38 kDa proteins compared with their non-diabetic counterparts (Figure 19). Analysing the IgG reactivity among groups that are matched for certain HLA molecules minimises the genetic variability. Because the Western blot data represent a single time-point at the time of diagnosis of T1D, we cannot confirm whether elevated levels of IgG to WG were present before clinical onset of the disease.

Low levels of antibody reactivity to dietary proteins such as cow's milk or gluten is common in healthy individuals [144]. However, increased entry of antigen as a result of damage to the intestinal mucosa may cause abnormal and pro-inflammatory local and systemic immune responses. We posit that the increased antibody reactivity to certain wheat proteins may be due to changes in immune regulation or loss of immunological tolerance to these dietary proteins in the GALT of pre-diabetic individuals. These WG

proteins may have a direct role in disease pathogenesis, possibly via antigen mimicry. Alternatively, the increased immunogenicity of these WG proteins in certain individuals may be a reflection of changes in immune regulation in the gut that affect islet homeostasis. The molecular characterisation of antigenic wheat proteins is needed to propose potential mechanisms to explain how ingestion of wheat promotes the development of diabetes.

Molecular characterisation of immunoreactive wheat proteins

Proteomics is the study of proteins using a high resolution separation technique, 2D gel electrophoresis combined with mass spectrometry, a sensitive microanalytical method for protein identification [145, 146]. The size distribution of wheat gluten proteins is such that several types of proteins of similar molecular mass co-migrate on a 1D-gel. Proteomics has been widely used in plant biology to study genetic diversity, phylogenetic relationships and characterise bioengineered variants [147]. The 2D Western blot analysis of wheat proteins showed that 11 proteins were immunogenic in at least 50% of BBdp rats (Table 4). These proteins were excised from 2D gels and analysed by mass spectrometry. The amino acid sequences the peptides obtained were used in a search of the NCBI sequence database and TIGR Wheat Gene Index for homologous amino acid sequences.

The immunogenic wheat proteins WG #4508, 5501, 5503, 5504 were identified as alanine aminotransferase-2 (barley GPT-2, Acc. No. P52894) in a query of the NCBI database and as alanine aminotransferase 2 transaminase 2 (wheat GPT-2, TC# 8197) in a query of the TIGR Wheat Gene Index. The protein source on our 2D gels was wheat, therefore the candidate proteins are wheat GPT- 2. The protein identification was based

on comparisons of protein fragment patterns obtained after trypsin digestion (theoretical and experimental digest), molecular mass and pI determination. Ten protein fragments after trypsin digestion were detected by mass spectrometry and matched the theoretical wheat GPT-2 proteins (TC# 8197, Table 5b). The immunogenic wheat GPT-2 proteins we detected using 2D-Western blots consisted of 4 isoforms of similar molecular mass, 65 kDa, but slightly different isoelectric point, pI ~6.3-6.5, which is similar to the expected values, 64 kDa and pI 6.33.

The identification of GPT-2 is noteworthy because elevated serum aminotransferase expression has been associated with gluten-sensitive enteropathy in children with T1D [148]. In addition, 46% (6/13) of BB rats with overt diabetes showed higher serum enzyme activity of glutamic pyruvic transaminase compared with the enzyme activity detected in asymptomatic BBdp rats and non-diabetic BBc animals [149].

We identified wheat proteins at WG #5406 as wheat storage globulin (WSG, Acc. No. AAA34269) in a query of the NCBI database. The experimental protein fragment pattern we detected after trypsin digestion of WG #5406 was consistent with the theoretical trypsin digest of WSG. Moreover, WSG proteins are oligomers composed of two protein subunits, 35 kDa (pI 6.9) and 49 kDa (pI 6.55) [128]. The WSG (WG #5406) we isolated from the 2D gels of wheat had a molecular mass of 50 kDa, pI 6.5 (expected 49 kDa, pI 6.55). We did not detect the 35 kDa (pI 6.9) globulin subunit on our 2D Western blots. However, it is possible that we detected the smaller globulin subunit in our analysis of 1D-Western blots. One explanation for these findings is that wheat proteins were reduced under different conditions in 1D Western blots (using β-mercaptoethanol)

and 2D Western blots (using tributyl phosphine, TBP and dithiothreitol, DTT). Based on the similarity in molecular mass and the strong association to T1D from cross-sectional and prospective 1D-Western blot analyses, we propose that the 36 kDa immunogenic wheat proteins may be the smaller globulin subunit (35 kDa).

Wheat gluten proteins, composed of gliadin and glutenin proteins, are the major storage proteins (~80%) in the wheat caryopse (wheat seed). These WG proteins are insoluble in water but soluble in aqueous alcohol (gliadins) and aqueous acetic acid (glutenins). WG is prepared commercially by ICN using an aqueous flour extraction to remove starch and some water-soluble proteins (albumins and globulins). Although, globulins are a minor constituent of the wheat seed, contributing 5-10% of the total protein content [150], only ~15% of wheat storage globulins are extracted in salt solutions (1.65 g of globulin proteins were extracted out of 11 g of total globulin proteins in seed meal) [151]. Therefore the majority of globulins will be present in the commercial extract of WG proteins.

It is interesting to note that globulins are the major protein constituent (90%) of soybean [152], a protein source that has been reported to promote development of diabetes in BBdp rats, albeit to a lesser extent than the WG diet [40]. Considering that we have identified WSG as a candidate immunogenic protein in wheat that may be associated with T1D, one explanation for the similar diabetes promoting potential of WG and soybean might be the presence of common immunogenic globulin proteins in both diets. 1D Western blots of soy proteins suggested that 11S glycinin proteins are immunogenic in individuals predisposed to T1D [53]. Our results suggest that the 11S glycinin G1 precursor (Acc. No. P04776) is 32% (10/31) identical in amino acid

sequence and 57% (18/31) similar in charge to WSG (Figure 7h). Moreover, one of the peptides isolated from the 2D gel, WG #5406 (peptide #12: VAIMEVNPR) is similar in 5/9 (55%) amino acids to 11S glycinin. It is possible that WSG and 11S globulins in soybean have common antigenic amino acid sequences.

The remaining 6 immunogenic wheat proteins (WG #4603, 4604, 4703, 4704, 5701 and 5505) could not be identified. There may be several reasons why we could not identify these proteins: 1) the mass spectrometry analysis yielded amino acid sequence data of insufficient length (less than 3 amino acids) to produce a meaningful database match; 2) the proteins in question may not be readily digestible by trypsin, which would impede mass spectrometry analysis; and/or 3) the proteins of interest are of low abundance and therefore there was an insufficient quantity of protein excised (less than 5 ng) from the 2D-gel for mass spectrometry analysis Skylas *et al.* have reported similar problems in obtaining amino acid sequences in ~30% of wheat-grain endosperm proteins (89/321 proteins analysed did not yield amino acid sequence data) [145]. Of the 1300 proteins they resolved by 2D-PAGE, approximately 300 proteins were identified by matching their amino acid sequence to the protein database at NCBI. Identification of wheat gluten proteins based on queries of the Swiss-Prot and TrEMBL databases from NCBI [79] are limited by the number of wheat sequences catalogued in these databases.

Sequence similarities between candidate wheat proteins and diabetes-related proteins

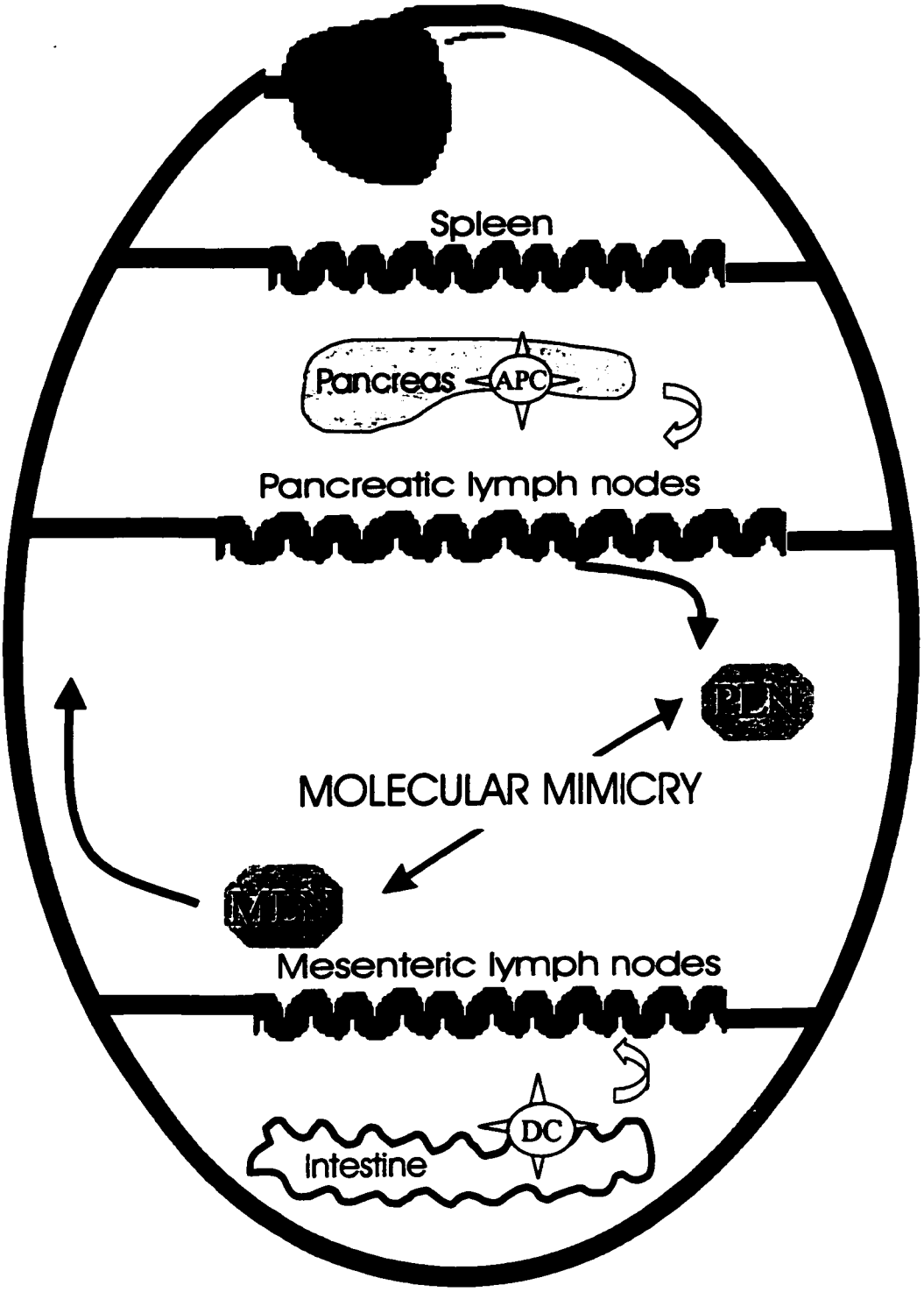
Alignment of the amino acid sequence of GPT-2 with those of diabetes-related molecules showed that it had 25% amino acid identity (29/112) and 41% of amino acids of similar charge (47/112) as human heat shock protein 60 (HSP 60, Acc. No. 129379, Table 7). Recently, a randomised, double-blind, phase II trial showed that immunisation

of patients newly diagnosed with diabetes with a peptide in HSP 60 (Diapep277) changed the profile of cytokines secreted by the patient's peripheral blood T cells from pro-inflammatory (high IFN-γ) to anti-inflammatory (high IL-4 and IL10) [153]. We found that the Diapep277 HSP60 peptide shares 57 % (4/7) amino acid identity and 85% (6/7) amino acids of the same charge with wheat GPT-2. The homologies between the candidate wheat peptides and the immunogenic human HSP60 peptide suggest the possibility of molecular mimicry (Figure 21). Presumably, T cells that recognise immunogenic wheat GPT-2 proteins and become activated have the potential to recognise similar amino acid sequences in HSP 60. β-cells expressing HSP 60 may become targets of these activated T cells leading to an autoimmune reaction.

Sequence alignment of WSG with diabetes related antigens showed that there is structural homology between WSG and human HSP60 Diapep277 (Table 7g). The sequence was identical at 10/30 amino acids (33%) and similar in 16/30 amino acids (53%) with respect to charge. Moreover, WSG also had amino acid homologies with (1) the insulin receptor precursor, which is related to insulin (the only β-cell specific autoantigen), and to (2) the IL-1 receptor and IL-1 beta, a β-cell cytotoxic cytokine. Sequence homology between WSG and IL-1 presents the possibility that the wheat protein may act as an agonist for the IL-1 receptor to stimulate a signalling cascade. The alignment of these sequences suggests that the potential exists for wheat proteins to stimulate an immune response that targets self-proteins or alters cytokine regulatory networks.

Figure 21 Model of molecular mimicry

Wheat proteins are ingested, subjected to digestion and then proteins and peptides of varying length transit through the gut lumen. It may be that epithelial cells can take up and present peptides but this point remains controversial. Some wheat proteins may cross the gut epithelium through microfold cells (M cells) that cover Peyer's patches or they can be sampled by dendritic cells from the lamina propria. These antigen presenting cells migrate to the mesenteric lymph nodes (MLN) where they may encounter naïve T cells that recognise certain wheat epitopes. The activated T cells migrate out of the MLN and travel via lymph to the thoracic duct which drains to the left subclavian vein. They migrate via the circulatory system throughout the body and may enter the pancreatic lymph nodes (PLN) through high endothelial venules. Antigen presenting cells traffic from islets of Langerhans in the pancreas to PLN and some will display β-cell specific antigens that stimulate T cells activated in the gut by lumenal antigens (wheat) that resemble the β-cell antigens.


Spleen
Pancreas
APC
Pancreatic lymph nodes
PLN
MOLECULAR MIMICRY
MLN
Mesenteric lymph nodes
DC
Intestine

WG proteins modify the development of T1D

Studies using animal models of spontaneous autoimmune diabetes, BBdp rats [39, 40] and NOD mice [41, 42] have shown that ingestion of a hydrolysed casein (HC) diet was diabetes-retardant, while a cereal-based standard rodent diet, NIH-07, promoted the development of T1D. Moreover, exposure to a wheat-based diet from weaning onward was a major modulator of the natural course of disease.

The gut of susceptible individuals may respond inappropriately to dietary WG proteins. Therefore we may have the potential to modify the course of the disease by inducing a state of hyporesponsiveness (oral tolerance) to WG proteins in these individuals. The most opportune time to induce oral tolerance is in the neonatal period because the gut is less well developed and the GALT is relatively immature [154, 155]. The outcome of local events in the GALT following exposure to WG proteins was examined in a study of 4-7 day old BBdp rats that were hand-fed twice daily with WG in Pregestimil, HC in Pregestimil or Pregestimil alone. The diabetes incidence in the group of rats fed WG in Pregestimil in the neonatal period was half that of rat pups fed Pregestimil alone (Figure 2 panel B). Moreover, onset of diabetes was delayed (82 vs 91 days) in animals that received early oral WG proteins. The data imply that stimulation of the GALT with diabetes-promoting wheat gluten proteins in the first week of life modified immune responses such that on exposure to wheat gluten at weaning progression to T1D was delayed or prevented in some animals. Similar findings were reported in NOD mice administered insulin in the neonatal period [156], suggesting that exposure to diabetes-relevant antigens in the neonatal gut may regulate autoimmune reactions in later life.

A caveat in studies aiming to induce oral tolerance to dietary or self-antigens is that such interventions can also induce autoimmunity depending on age and dose of antigen [157]. This disparity may be attributed to differences in the physiological and immunological environment at the time of early oral dosing. Notably, the gut mucosa is more permeable in neonatal humans [158] and rats [159] compared to adults. In addition, exposure to the diabetes-promoting dietary components in the first week of life occurred in the presence of dam's milk. Dam's milk provides growth factors, cytokines and other immunomodulatory peptides that may be essential for "normal" GALT development and subsequent hyporesponsiveness to diabetes-promoting macromolecules from the gut lumen.

The GALT is exposed to a mixture of antigens and their breakdown products. Normally, the GALT is hyporesponsive to dietary antigens that cross the gut epithelium. However, in some individuals, recognition of certain dietary antigens primes the immune system and causes a disproportionate immune reaction, a hypersensitivity response.

In allergy or type 1 hypersensitivity, sufficient numbers of IgE antibodies bind to certain dietary macromolecules and initiate the complement cascade that results in mast cell degranulation. Such is the case in Baker's asthma, where individuals develop IgE mediated asthma from exposure to water/salt soluble wheat allergens [96]. A classic example of a "food-induced" gut enteropathy is celiac disease, a type 4 hypersensitivity. Individuals with certain disease risk haplotypes characterised by antigen presenting molecules, HLA-DQ2 and HLA-DQ8 MHC class II genes, may develop intestinal inflammation upon ingestion of wheat proteins [160]. Avoidance of these proteins in the diet leads to the disappearance of inflammation and clinical symptoms. Re-challenge of

these patients with the offending proteins provokes crypt hyperplasia, flattened villi and increased numbers of intraepithelial lymphocytes in the gut.

Of interest is the observation that there is a high frequency of patients with T1D who also have overt celiac disease (5-10%), which is 17-33 times higher than in the general population. Consistent with these observations, higher levels of antibodies to tissue transglutaminase, a recently discovered celiac autoantigen, were detected in patients with T1D. It was reported that 10% of patients with T1D had similar levels of transglutaminase C antibodies as patients with celiac disease and an additional 30% had low transglutaminase C antibody binding [72]. The prevalence of CD in patients with T1D was 5.7% as determined by serological tests for anti-endomysial antibody as well as intestinal biopsy, which identified patients with subclinical/silent CD [104]. By contrast, the frequency of CD in patients with type 2 diabetes (non-insulin dependent diabetes) is similar to the general population, ~0.3% [161]. These statistics suggest that gut enteropathy and anti-islet immune reactivity frequently occur in parallel and may have common origins. Another interesting point is that diagnosis of CD is more frequent after diagnosis of T1D but it is rare to observe the clinical onset of diabetes after CD has been confirmed [162]. One explanation for this observation may be that the wheat gluten free diet prescribed following the diagnosis of CD also prevents diabetes. Thus, we hypothesise that wheat may be involved in the pathogenesis of T1D, possibly by inducing a subclinical gut inflammation and stimulating β-cell-specific T cells, which promotes the development of diabetes in some individuals. In addition, prior to the development of insulitis, diet may also modify β-cell growth and metabolism, thus affecting islet mass, antigenicity and insulin reserves.

Cow milk proteins have been reported as another potential source of diabetes promoting dietary antigens [12]. One hypothesis proposed that antibodies to the ABBOS peptide in milk were cross-reactive with islet-cell p69 antigen (ICA69) and therefore could initiate and perpetuate the autoimmune response. Another explanation may be related to the status of the gut epithelial barrier following exposure to milk proteins. It was reported that early (at day 14) exposure to cow's milk proteins interferes with the maturation of the rat gut, leading to increased jejunal permeability (delayed gut closure) and infiltration of eosinophils [140]. The increased mucosal permeability was attributable to a local hypersensitivity reaction to cow milk proteins. A prolonged permeability of the gut and activation of pro-inflammatory immune responses in the neonate may increase antigen load. As a result, immune responses may spread to unrelated dietary proteins. Suomalainen *et al.* showed that exposure to cow's milk proteins in patients with cow's milk allergy prompted immune responses to unrelated antigens such as gliadin proteins from wheat [163]. Hence the outcome of early administration of dietary antigens may establish oral tolerance or hypersensitivity, depending on the age and immune status of the individual.

For ethical reasons, feeding studies carried out in rodents cannot be repeated in humans. The only prospective nutrition intervention trial in humans is the Trial to Reduce IDDM in the Genetically at Risk (TRIGR), a double blind, prospective placebo-controlled study. It will examine whether avoidance of intact cow's milk proteins during the first 6 months of life will delay or prevent the development of autoimmunity and T1D in children with HLA alleles associated with higher risk of diabetes or first-degree relatives of patients. The preliminary results suggest that fewer islet cell autoantibodies

were detected in high risk Finnish infants that were breastfed or received a casein hydolysate (Nutramigen) in the first 6-8 months of life (avoided cow milk) than in infants fed a cow's milk based formula [52]. Moreover, infants that avoided cow's milk proteins had a lower incidence of autoimmunity (as defined by ICA, GAD, IAA, IA-2) compared with those receiving cow's milk protein-based formula. Following these encouraging results, the study will now aim to determine whether early avoidance of cow's milk protein will reduce the frequency of T1D.

However, changes to infant feeding practices should be undertaken with caution. The National Board of Health and Welfare in Sweden recommended in 1973 that the introduction of wheat be postponed from the first week of life to 4 months after birth, and in 1983 this was extended to 6 months. In addition, the dose of gluten in infant formula was doubled in the 1980s. Following these changes to infant feeding practices, there was a three-fold increase in the incidence of CD [164]. The temporal association of these observations suggests a potential risk in changing infant diet formulations and practices on the development of disease. Therefore, changes to recommendations for infant feeding are ill-advised at present until the link between diet and autoimmunity is better understood, particularly the identity of dietary antigens that modulate diabetes outcome.

The GALT is a major interpreter of dietary information, a function that has been used to modify immune responses to autoantigens. Induction of oral tolerance to one of the diabetes autoantigens, GAD, expressed in transgenic tobacco plants was successful in reducing the incidence of diabetes in NOD mice [89, 165]. Currently there is a clinical trial underway called the Diabetes Prevention Trial-1 (DPT-1) to examine whether administration of tolerizing doses of insulin via the oral or intravenous route to children

at risk of diabetes will reduce the incidence of T1D [166]. The results from the first part of the trial suggest that insulin injection did not reduce T1D. Results from oral administration of insulin are not yet available. A preliminary study to obtain complementary results showed that oral administration of insulin did not prevent β-cell destruction [167]. Although, the initial results from the clinical trial and the animal studies are not consistent, this may be attributed to different confounding factors associated with the respective studies. Humans, unlike laboratory animals are not confined to a constant environment and so other factors such as time of treatment, immune status and infection will influence the success of these oral interventions. These findings further emphasise the need for a better understanding of the mechanisms of immune regulation in the gut and a more complete knowledge of environmental influences on the development of T1D.

It was reported in NOD mice that some of the lymphocytes in the inflamed pancreas display $\alpha_4\beta_7$ integrins, suggesting that they originate from the gut mucosa [168]. As many as 10% of gut lymphocytes normally traffic between the GALT and the pancreas via lymph vessels. Data showing that cells isolated from mesenteric lymph nodes can transfer diabetes have been reported [169], suggesting that gut immune cells may be direct participants in the development of T1D. Similarly, others have reported that blocking mucosal adhesion molecules and integrins using anti-MAdCAM-1 and anti-$\alpha_4\beta_7$ antibodies prevented diabetes [170]. The implication is that the development of diabetes involves mucosal lymphocytes expressing $\alpha_4\beta_7$ integrins that home to their receptor, MAdCAM-1, expressed in the pancreas. Therefore, the status of mucosal

lymphocytes, that is the cytokine profile they secrete and the ligands they express on their cell surface, may be of critical importance to how they affect islet cells.

In our studies we showed that cytokine patterns in the gut of rat pups were modified by diet. We examined changes in cytokine levels in duodenal sections of the small intestine of 5-day-old rat pups 24 hours after exposure to Pregestimil or the cereal based NIH-07 diet in Pregestimil. We detected a lower ratio of IFN-γ /TGF-β in NIH-07 fed rats compared with PG fed animals [171]. This is consistent with our hypothesis that there may be a breakdown in regulatory mechanisms in the gut of BBdp rats that normally prevent Th1 responses to dietary proteins. By corollary, the downregulation of a pro-inflammatory response in the gut may prevent T1D. The decreased level of IFN-γ may reflect a shift from Th1 towards Th2 immune responses. Exposure to diabetes promoting proteins in neonates has the potential to modify local immune responses to dietary antigens. The events in the gut and pancreas that contribute to disease progression remain to be defined.

Development of an in vitro test system of food protein "diabetogenicity"

As dietary proteins and their degradation products transit through the gastrointestinal track they may come in contact with epithelial cells. Recently *in vitro* studies with HT-29 epithelial cells, which mimic the epithelium in the small bowel, demonstrated that these epithelial cells had an active role in processing dietary proteins to immunologically relevant peptides [142]. Moreover, enterocytes respond to changes in dietary factors, which has several advantages. First, upregulation of enzymes or transporters in the intestinal epithelium in response to repeated exposure to some dietary agents improves digestion and absorption of these nutrients. Second, growth and immune

factors found in breast milk confers information about the environment and influences the maturation of the gut epithelium. Third, enterocytes convey information about potential pathogens in the gut lumen to the GALT. Therefore, changes in the activity of enterocytes in response to dietary antigens may reflect the biological activity of wheat proteins. We designed an *in vitro* test system to study the effect of digested WG proteins on protein expression in intestinal epithelial cells (Appendix II).

A cell line of rat intestinal epithelial cells was incubated in the presence of digested wheat gluten proteins and following three days of culture, the cell lysate was analysed using proteomic analysis. The 2D gels of the cell lysate indicated that there was a major down regulation in protein expression in gut epithelial cells following exposure to diabetes-promoting proteins (WG and soy), similar to the response seen in the small intestine of celiac patients challenged with WG [172]. Protein expression was enhanced in the presence of HC. By contrast with WG and soy treatments, cells exposed to HC expressed antioxidant enzymes such as thiol-specific antioxidant protein 2 and thioredoxin peroxidase, as well as Annexin V and galactokinase (Appendix II, Table A). Heat shock protein 40 and certain enzymes were absent or down regulated in cells cultured with WG or soy. A similar decrease in the levels of heat shock proteins was observed in the jejunal tissue sections of rats fed wheat germ agglutenin [173]. Our findings suggest that exposure to HC induced an antioxidant response. The pathogenesis of diet-induced T1D may be related in part to a differential response of the gut epithelium to diabetes-promoting proteins, a point that requires further analysis. Proteomic analysis of IEC-6 cells is a useful tool to begin to evaluate the interaction between these dietary agents and the gut epithelium.

CONCLUSION

Feeding wheat gluten proteins from weaning at ~23 d promoted the development of diabetes, whereas exposure to a mixture of cereal proteins and WG alone in the first week of life resulted in incidence of disease that was less than one third to one half that of animals not exposed to these oral antigens as neonates. The decrease in the ratio of IFN-γ /TGF-β gene expression 24 hours after ingestion of these cereal antigens showed that the immune response in the GALT was modified according to the dietary antigens in the lumen. This shift in cytokine profile was consistent with a downregulation of pro-inflammatory responses and may help explain why this intervention resulted in a delay of T1D onset and fewer diabetes cases. In addition, susceptible individuals show different immune reactions to wheat gluten proteins. Pre-diabetic BB rats and Finnish children newly diagnosed with diabetes showed higher IgG reactivity to 33, 36 and 38 kDa wheat proteins. 2D Western blot and mass spectrometry analysis identified two immunogenic candidate wheat proteins, alanine aminotransferase 2 transaminase 2 and wheat storage globulin. The peptides identified as GPT-2 share structural homology with one of the autoantigens in diabetes, heat shock protein 60. Moreover, peptides identified as the WSG have amino acid homologies with the insulin receptor precursor and IL-1 receptor, which are related to insulin (a β-cell specific autoantigen) and the β-cell cytotoxic cytokine, IL-1 beta, respectively. We propose that the gut plays a role in the pathogenesis of diabetes in BB rats, possibly as a source of inflammatory cells that travel to the pancreas and attack insulin-secreting β-cells and also as a major contributor of information that controls the mass and function of insulin-containing cells in the pancreas. The proteomic analysis of immunogenic wheat proteins is the first direct

molecular characterisation of dietary wheat proteins that may be related to the development of T1D. Further characterisation of their function in diabetes pathogenesis may lead to changes in infant feeding practices, new diagnostics and ways to delay or prevent disease by modifying the gut immune system.

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Appendix I: Outliers

Western blots of wheat gluten proteins probed with sera from BB rats or non-diabetic Finnish children that could not be analysed. Individual protein bands could not be identified because signal intensity was too high.

84F 86F Finnish control children

Appendix II: Development of an in vitro test system

Rationale

Lumenal antigens that transit through the gut epithelium come in contact with the gut epithelial cells. Information encoded in dietary antigens is interpreted by enterocytes, a process which in turn may be reflected in changes in enterocyte protein expression We examined the effect of incubating gut epithelial cells in the presence of dietary antigens with different diabetes-promoting potential (HC, WG, soy) to develop an *in vitro* system for testing the potential of dietary antigens to promote changes in the gut epithelium.

Methods

WG protein digestion: WG proteins, which are insoluble in physiological salt solutions, were made soluble through enzymatic proteolysis as described by Arentz-Hansen *et al.* [174, 175]. In brief, 1.0 g of WG flour was digested with α-chymotrypsin (Sigma Cat. No. C-4129; 1:100 w/w) overnight at 37 °C in 0.174M Tris-base (Sigma) at pH 7.8. The peptide suspension was centrifuged at 15000g (Eppendorf Centrifuge 5417R, Brinkmann Instruments, Inc., Mississauga, ON) for 15 min at room temperature. The supernatant was diluted 1:1 with Dulbecco's phosphate buffered saline (PBS, Life Technologies, #21600-010) and filtered through a 1.2 μm Acrodisc Syringe filter (Supor Membrane, Pall Gelman Laboratory, Ann Arbor, Mi, U.S.A.) and then sterile filtered through a 0.2-μm Acrodisc Syringe filter. The protein concentration was determined using the BioRad protein reagent. Aliquots of the protein digest were stored at –20°C.

Cell lines: An adherent cell line of normal rat epithelial cells from the small intestine (IEC-6) was purchased from the American Type Culture Collection (ATCC #CRL 1592,

Manassas, VA, U.S.A.). These are non-differentiating cells that express antigens characteristic of intestinal epithelia. Cells were cultured in Dulbecco's Modified Eagle's Medium (ATCC #30-2002; 4 mM L-glutamine, 4500 mg glucose/L, 1500 mg sodium bicarbonate/L) supplemented with 2.1 mg bovine pancreatic insulin (Sigma I1882), 1.1 mM L-glutamine (Life Technologies, GibcoBRL #25030-081, Grand Island, NY, U.S.A.) and 10% fetal calf serum (Sigma F1051). Cells were split twice weekly at a ratio of 1:6 using trypsin-EDTA (Life Technologies #25200-056, 0.25% trypsin and 1 nM EDTA·4Na) to dislodge the monolayer. In IEC-6 cell cultures stimulated with 50 μg/ml of dietary antigens (digested WG, soy or hydrolysed casein) or unstimulated control cultures, the medium was supplemented with 100 units/ml penicillin G sodium and 100 μg/ml streptomycin sulfate in 0.85% saline (Life Technologies #15140-122).

Cell proliferation: Viable cell count was performed using Trypan blue exclusion. The cell suspension was mixed with trypan blue stain (0.4%, Life Technologies #15250-061) in a 1:3 ratio and the number of cells that excluded the dye (translucent) as well as those in which the dye had penetrated the cell (blue) were counted in a haemocytometer using a Zeiss microscope and 20x objective. For cells cultured in 96-well plates, cell viability was determined using the cell proliferation reagent WST-1 (Roche Diagnostics, Laval, QC, Canada). The WST-1 reagent is converted to formazan by viable cells, a chromogenic product that can be read on a microplate reader (BioRad) at 450 nm with a 655 nm reference wavelength.

2D cell lysate: IEC-6 cells were seeded at a density of 10^5 cells/ well of a 6-well plate. One set of wells contained medium alone, while another set received medium and 50 μg/ml chymotrypsin digested wheat gluten proteins. Cells were cultured in 5% CO_2 for 72 hours. Cells were harvested, washed with cold PBS (Life Technologies) and counted using Trypan Blue exclusion dye (Life Technologies). The cell lysate was analysed by 2D gel electrophoresis using a modified protocol described by Genomic Solutions Inc. [176]. In brief, 10^6 cells from each treatment group were transferred to a fresh tube and centrifuged at 4°C for 5 min at 21,000 g. Cells were lysed by suspending them in 500μl of rehydration buffer (4% CHAPS, 7M urea, 2M thiourea, 40 mM Tris-base, 2mM tributylphosphine (TBP) and 0.4% Biolyte 3-10). Cell suspensions were sonicated for 5 min at room temperature in a water bath and the cellular debris was pelleted by centrifugation at 4°C for 5 min at 21000 x g (Eppendorf Centrifuge 5417R, Brinkmann Instruments, Inc.). 400μl of the supernatant were transferred to a fresh tube and 4μl of TBP and 4μl of Biolyte were added to the cell lysate. 300μl of cell lysate were pipetted onto the rehydration tray for passive rehydration of IPG strips pI 3-10, overnight at room temperature. The 2D gels of the cell lysate were prepared as described above for wheat gluten proteins (see 2D Westerns). The 2D gels were stained with non-reducing silver stain [116]. An image of the 2D gel was obtained using the Kodak image station and analysed by 2D software (PDQuest, BioRad). Proteins detected in the various treatment groups were compared. Proteins that were detected in one treatment group but not the other were excised for mass spectrometry analysis.

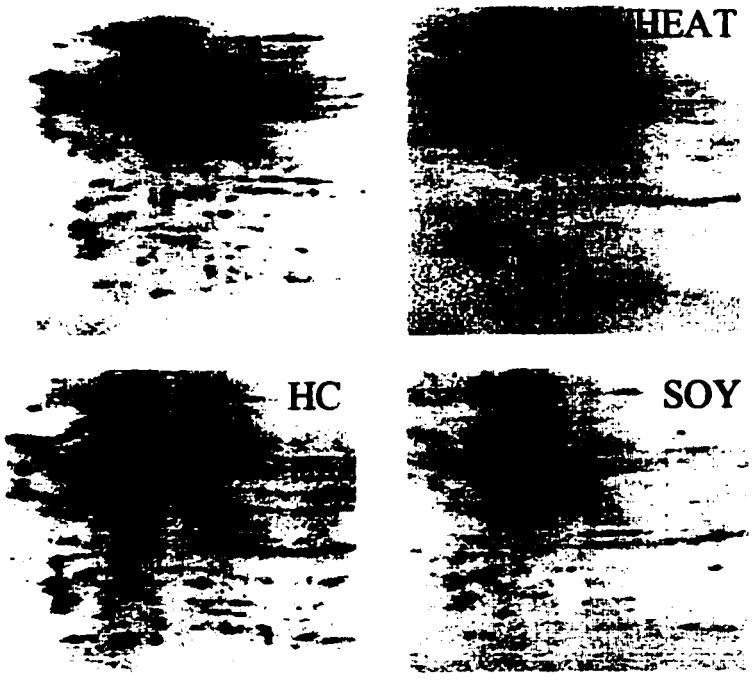
Results:

Once we identified the antigenic wheat gluten proteins we needed a test system to demonstrate that these proteins would have biological consequences. Cell lysate proteins of gut epithelial cells stimulated with dietary antigens were resolved using 2D electrophoresis to get an overall impression of protein expression. Others studying gluten-sensitive enteropathy have done similar 2D-gel analyses to characterise the jejunal microvillar membrane proteins [177]. Appendix II Figure A shows the cell lysate of IEC-6 cells incubated with wheat gluten proteins. There was a general down regulation in protein expression in IEC-6 cells incubated with diabetes-promoting dietary proteins, WG and soy. The presence of HC produced a protein pattern similar to control unstimulated IEC-6 cells. The fact that protein expression appeared to be related to the diabetogenic potential of the protein source suggests that diabetes-promoting proteins could have different effects on representative gut epithelial cells. We examined the differences in protein expression by PDQuest (BioRad) and the proteins whose expression was different as a consequence of the various treatment groups were analysed by mass spectrometry. Appendix II Table A summarises the changes in protein expression. Unlike WG and soy stimulated cells, IEC-6 cells exposed to HC expressed antioxidant enzymes such as thiol-specific antioxidant protein 2 and thioredoxin peroxidase, as well as Annexin V and galactokinase (Appendix II, Table A). Heat shock protein 40 and certain enzymes were absent or down regulated in cells cultured with WG or soy. The response induced by diabetes promoting WG and soy proteins is suggestive of gut damage, while HC may promote an antioxidant response. These studies provide proof of principle that *in vitro* test systems can be employed to evaluate nutrient-protein expression in target tissues relevant to diabetes pathogenesis.

Appendix II Table A: Nutrient-protein expression system

Untreated IEC-6	WG treated IEC-6	SPI treated IEC-6	HC treated IEC-6
Proteasome delta chain			
Antioxidant protein 2			
Proteasome beta chain			
Phosphoglycerate mutase	Phosphoglycerate mutase		
Glutathione S- transferase P	Glutathione S- transferase P		Glutathione S-transferase P
Annexin III			
Malate dehydrogenase			
Annexin I	Annexin I		
ER-associated HSP40			
Reticulocalbin 1			
Eukaryotic translation elongation factor 1			Eukaryotic translation elongation factor 1
	Annexin II	Annexin II	
	Glyceraldehyde-3- Phosphate dehydrogenase	Glyceraldehyde-3- Phosphate dehydrogenase	Glyceraldehyde-3- Phosphate dehydrogenase
		Phosphatidyl ethanolamine-binding protein	
			Thiol-specific antioxidant protein 2
			Thioredoxin peroxidase
			Guanine nucleotide-binding protein beta
			Annexin V
			Galactokinase

Appendix II Figure A: Rat intestinal epithelial cells (IEC-6) incubated with chymotrypsin digested wheat gluten proteins, soy or hydrolyzed casein (HC). Protein expression was compared using 2D gels of cell lysate.


HEAT
HC
SOY

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Undergraduate Entrance Award	Sept. 1993 University of Ottawa $1000

TRAVEL BURSARIES:

September 9-13, 2001	Department of Biochemistry/ Microbiology/Immunology, Faculty of Medicine, University of Ottawa 37th European Association for the Study of Diabetes conference, Glasgow, Scotland. $1000
June 21-24, 2000	Canadian Society of Nutritional Sciences Canadian Federation of Biological Sciences conference, Ottawa, Canada $150
November 13-15, 1999	Immunology of Diabetes Society 4th Immunology of Diabetes conference, Fiuggi, Italy. $1000 US

PUBLICATIONS:

Karolina M. Burghardt, Amanda J. MacFarlane, John Kelly, Olli Simmell, Tulla Simell, Illimar Altosaar and Fraser W. Scott. Molecular characterisation of candidate wheat proteins associated with type 1 diabetes (In preparation)

Isabel Valverde, Gen-Sheng Wang, **Karolina Burghardt**, Araceli Redondo, Alicia Acitores, Maria L. Villanueva-Peñacarrillo, Phillippe Courtois, Abdullah Sener, Willy J. Malaisse and Fraser Scott. Low expression of gut proglucagon mRNA, GLP-1 protein and its receptor in the pancreas of diabetes-prone rats. (Submitted to *Journal of Endocrinology*)

Karolina M. Burghardt and Fraser Scott. Immunology of Food. In: Encyclopedia of Food Sciences and Nutrition. Eds. B. Caballero, L.C. Trugo and F.M. Finglas. Academic Press, London (In Press, March 2003)

Fraser W. Scott, Paul Rowsell, Gen-Sheng Wang, **Karolina Burghardt**, Hubert Kolb, Stefanie Flohé. Oral exposure to diabetes-promoting food or immuno-modulators in neonates alters gut cytokines and diabetes. *Diabetes*. 51(1): 73-78, 2002.

Bellmann K, Kolb H, Hartmann B, Rothe H, Rowsell P, Rastegar S, **Burghardt K**, Scott FW. Intervention in autoimmune diabetes by targeting the gut immune system. *Int. J. Immunopharmac.* 19(9/10): 573-577, 1997.

ABSTRACTS AND ORAL PRESENTATIONS: (* recipient of travel award)

* September 9-13, 2001 European Association for the Study of Diabetes
Karolina M. Burghardt, John Kelly, Tammy-Lynn Tremblay, Pierre Thibault, Fraser Scott. Diabetes-promoting dietary proteins change protein expression in rat intestinal epithelial cells.

* September 9-13, 2001 European Association for the Study of Diabetes
A.J. MacFarlane, **K.M. Burghardt**, R. Sardana, I. Altosaar and Fraser Scott. Immunoscreening a wheat cDNA library for candidate peptides involved in the pathogenesis of type 1 diabetes.

June 21-24, 2001 Canadian Federation of Biological Societies
Karolina M. Burghardt, John Kelly, Tammy-Lynn Tremblay, Pierre Thibault, Fraser Scott. Proteomic analysis of rat intestinal epithelial cells exposed to dietary peptides of different diabetes promoting potential.

May 26, 2001. Invited speaker to the JDF Group Meeting/Workshop: Gut dysfunction, diet and autoimmune diabetes, Brussels, Belgium.
Proteomics to study diabetes related wheat proteins.

* June 20-25, 2000 Canadian Federation of Biological Societies
Karolina M. Burghardt, Olli Simell, Tuula Simell, Fraser Scott.
Antibody reactivity to wheat gluten proteins in BB rats at risk of developing type 1 diabetes

* Nov 10-15, 1999 4th Immunology of Diabetes Society Congress
K.M. Burghardt, O. Simell, T. Simell, F.W. Scott.
Distinct antibody reactivity to wheat gluten proteins in BB rats at risk of developing type 1 diabetes and newly diabetic Finnish children

* Oct. 13-16, 1999 Canadian Diabetes Association Conference
Karolina M. Burghardt, Olli Simell, Tuula Simell, John Kelly, Pierre Thibault, Fraser Scott.
An approach to identifying diabetes related wheat gluten proteins using Western blotting and mass spectrometry

* Nov. 5 1998 seminar guest speaker at Diabetes-Foschungs Institut, Heinrich-Heine University of Düsseldorf, Düsseldorf, Germany. Immune response to dietary wheat gluten proteins in type 1 diabetes.

July 14-18, 1997 4th Lessons from Animal Diabetes Conference:
Experimental and Clinical Endocrinology and diabetes.1997, 105(Suppl. 3), 24.
Scott FW, **Burghardt KM**, Rastegar S, Zegers A, Jee P.
Oral agents encountered in infancy modify control of diabetes outcome by weaning diets in BB rats.

May 1997. 1st European Conference on Immunopharmacology
Kolb H, Hartmann B, Bellmann K, Rothe H, Jee P, Rastegar S, **Burghardt K**, Scott FW.
Intervention in autoimmune diabetes by targeting the gut immune system.

TEACHING AND TRAINING:

Sept. 2000-April 2001: Co-supervision of student project for undergraduate Biochemistry Honour's thesis at the University of Ottawa
Sylvia Balabanian, **Karolina Burghardt** and Fraser Scott
Title: Culture of BB rat lymphocytes with gut epithelial cells in the presence of food antigens

January 16 2001: Lecturer to 1st year medical students at University of Ottawa
Title: Breakdown in tolerance and hypersensitivity reactions

Oct. 23-Nov. 6, 1998: Training work term at the laboratory of Professor Dr Hubert Kolb, Immunology and Molecular Genetics, Diabetes Research Institute, Heinrich-Heine University of Düsseldorf, Düsseldorf, Germany as part of Canada-Germany Scientific Exchange Program.

Jan. 23-30, 1997: Training work term at the laboratory of Professor Dr Hubert Kolb, Immunology and Molecular Genetics, Diabetes Research Institute, Heinrich-Heine University of Düsseldorf, Düsseldorf, Germany as part of Canada-Germany Scientific Exchange Program.

LANGUAGES:

Scale: 1 (basic) to 3 (fluent)
Reading, Writing, Speaking
English: (3, 3, 3)
French: (3, 3, 3)
Polish: (2, 2, 3)
Spanish: (2, 1, 1)

STATEMENT OF CONTRIBUTION OF COLLABORATORS

Dr John Kelly

Mass spectrometry analysis was performed at the National Research Council of Canada in the laboratory of Dr Pierre Thibault by Dr John Kelly.

Dr Stefanie Flohé

The analysis of gut cytokines by RT-PCR was performed at the Department of Immunology and Molecular Genetics, Diabetes Research Institute, Heinrich-Heine University of Düsseldorf, Düsseldorf, Germany, in the laboratory of Professor Dr Hubert Kolb by Dr Flohé.