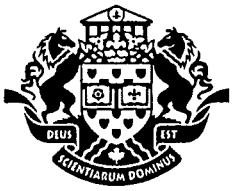

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FACULTÉ DE ÉTUDES SUPÉRIEURES ET POSTDOCTORALES

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Identification and Characterization of the type 1 Diabetes-related to Wheat Storage Globulin WP5212

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LE DOYEN DE LA FACULTÉ DES ÉTUDES SUPÉRIEURES ET POSTDOCTORALES

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Identification and Characterization of the Type 1 Diabetes-Related Wheat Storage Globulin WP5212

Amanda J. MacFarlane

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies
In partial fulfillment of the requirements
For the Ph.D. degree in Biochemistry

Department of Biochemistry, Microbiology and Immunology
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ABSTRACT

Type 1 diabetes (T1D) affects approximately 200,000 Canadians and is increasing at a rate of about 3.0% per year. The development of autoimmune diabetes requires genetic susceptibility and is influenced by environmental agents. Diets in which wheat protein is the sole amino acid source are associated with high diabetes frequency in diabetes-prone BioBreeding (BBdp) rats and non-obese diabetic (NOD) mice, two models of spontaneous autoimmune diabetes. We hypothesized that an abnormal immune response to wheat, as indicated by circulating IgG antibodies, could be used to identify T1D-related wheat proteins. Similar methods have been used to identify the diabetes-related autoantigens.

A wheat cDNA expression library was immunoscreened with pooled IgG antibodies from wheat gluten-fed diabetic BBdp rats. Initially, three clones were isolated. When the proteins were screened with sera from individual rats at varying risk of disease, antibody reactivity to clone WP5212, a wheat storage globulin, was strongest in diabetic rats. This antibody reactivity was not only associated with disease but also closely correlated with islet damage. Additional studies revealed that WP5212 antibodies were linked to diabetes in NOD mice and, remarkably, in human T1D patients. Furthermore, patients with both T1D and the wheat sensitive enteropathy, celiac disease (CD), displayed high WP5212 antibody reactivity, possibly representing a marker of the predisposition of T1D patients to develop CD.

WP5212 shared homology with a number of dietary proteins and allergens, suggesting that diabetes-promoting structures could exist in other food plants and that immunomodulatory molecules could interact with the immune system by a common

mechanism. WP5212 also shared homology with self proteins, which could act as a means for the initiation or promotion of autoimmunity.

WP5212 is the first candidate dietary wheat protein implicated in the development of diabetes. Thus, screening a cDNA expression library provides a novel approach that permits the identification of environmental antigens involved in autoimmune diseases. The evidence suggests that WP5212 is strongly antigenic in three separate species that develop spontaneous diabetes. The molecular identity of this immune-targeted dietary wheat protein will help elucidate the pathogenic mechanisms by which wheat-related diabetes occurs.

DEDICATIONS

To all of my friends and family who have supported me in my studies over the past few years; I couldn't have done it without you. And to Alex, without you it would not have been possible.

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LIST OF ABBREVIATIONS (In alphabetical order)

Abbreviation	Definition
ANOVA	Analysis of variance
AP	Alkaline phosphatase
APC	Antigen presenting cell
APS	Ammonium persulfate
BBc	Control BioBreeding rat
BBdp	Diabetes-prone BioBreeding rat
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
CBV	Coxsackie B virus
CD	Celiac disease
cDNA	Complement deoxyribonucleic acid
cfu	Colony forming units
CIAA	Chloroform: Isoamyl alcohol
CMV	Cytomegalovirus
DC	Dendritic cell
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
EV	Enterovirus
GAD(A)	Glutamic acid decarboxylase (antibody)
GFAP	Glial fibrillary acidic protein
H&E	Hematoxylin and eosin
HC	Hydrolyzed casein
HLA	Human leukocyte antigen
IA-2	Protein tyrosine phosphatase-like IA-2/IA-2β
IAA	Insulin autoantibody
ICA	Islet cell antigen
IFNγ	Interferon γ
IgG	Immunoglobulin G
IL	Interleukin
IPTG	Isopropyl β-thiogalactopyranoside
IVGTT	Intravenous glucose tolerance test
LB	Luria broth
LSD	Least significant difference
MAdCAM-1	Mucosal addressin cell adhesion molecule-1
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
NBT	Nitroblue tetrazolium
NIH	National Institutes of Health (NIH)-31 (6% fat) cereal-based diet
NK	Natural killer

NOD	Non-obese diabetic
NOR	Non-obese resistant
NTP	National Toxicology Program (NTP)-2000 wheat based diet
NZY	NZ amine
OD	Optical density
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PDB	Protein Databank
pfu	Plaque forming units
PMSF	Phenylmethylsulfonyl fluoride
RNA	Ribonucleic acid
SD	Standard deviation
Sf21	*Spodoptera frugiperda*
T1D	Type 1 diabetes
TBS	Tris-buffered saline
TBST	Tris-buffered saline + 0.1% Tween20
TE	Tris-EDTA
TEMED	N,N,N′,N′-tetramethylethylenediamine
Th	T helper lymphocyte
TIGR	The Institute for Genomic Research
TNF	Tumor necrosis factor
tTG	Tissue transglutaminase
WG	Wheat gluten
WP	Wheat protein
WP12111	Wheat cDNA clone 12111
WP23112	Wheat cDNA clone 23112
WP5212	Wheat cDNA clone 5212
WPCON	Negative control wheat cDNA clone
X-gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside
1D SDS-PAGE	One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis

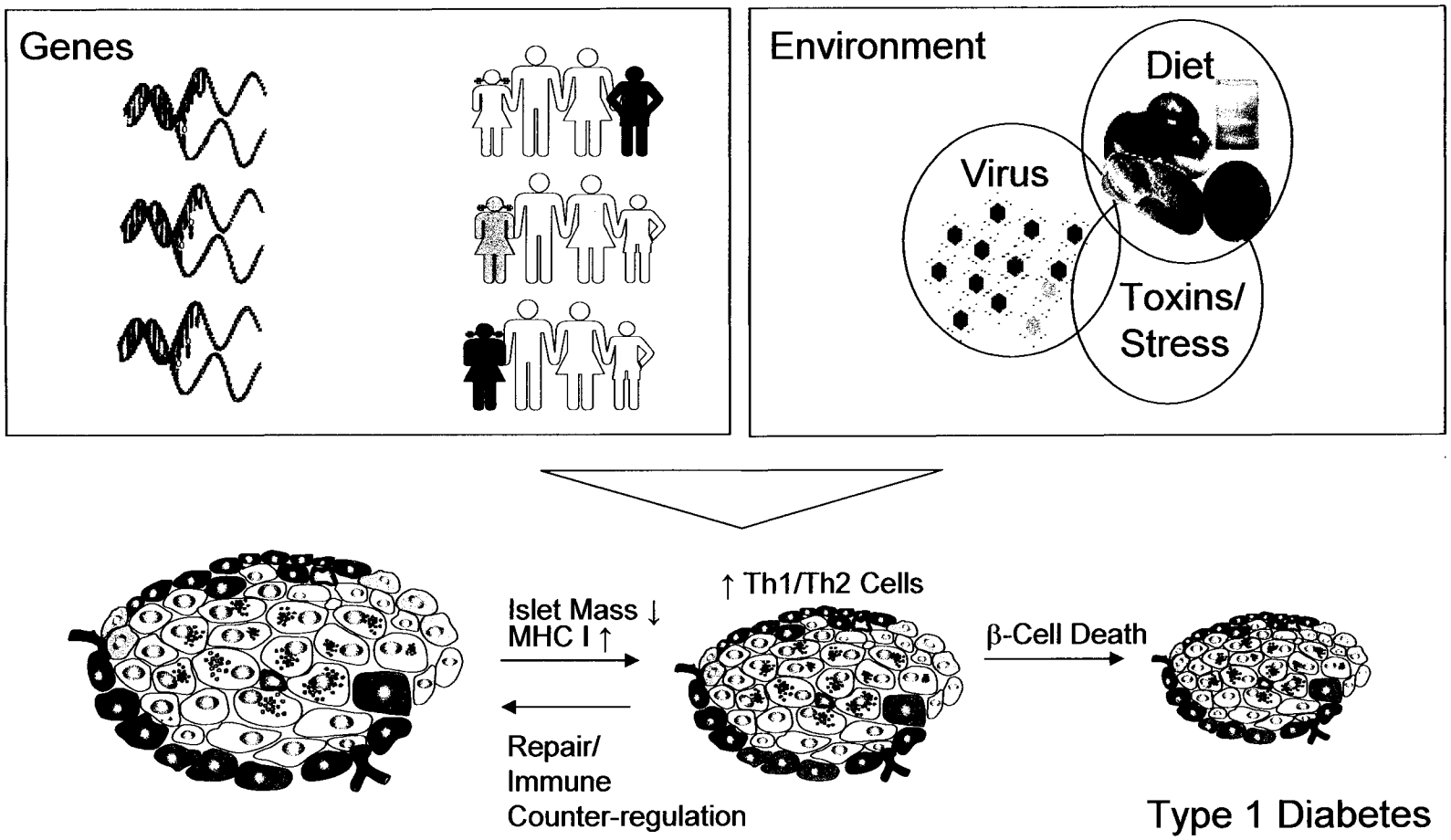
INTRODUCTION

Type 1 diabetes (T1D) is one of the most common chronic diseases of childhood affecting approximately 0.6% of the Canadian population (http://www.diabetes.ca/Section_about/prevalence.asp, accessed 20/07/04). T1D is a spontaneous autoimmune disease resulting from the immune-mediated destruction of the insulin-secreting β-cells in the pancreatic islets of Langerhans (1). The pathogenesis of this disease is complex requiring the interaction of both genetic susceptibility and, as yet unknown environmental factors for disease development (Figure 1). With the right combination of these factors, an inflammatory immune attack is activated that specifically targets the β-cells. The body can counteract this attack by mechanisms of islet repair and by immune counter-regulation. However, the inflammatory process eventually overcomes this counter-regulation resulting in a critical reduction of β-cell mass and the loss of glucose homeostasis. A life long dependency on exogenous insulin ensues. Complications of T1D include cardiovascular disease, nephropathy, neuropathy, retinopathy, limb amputation, and an average decrease in life expectancy of 15 years. Unfortunately, there is currently no preventive therapy or cure for autoimmune T1D.

Epidemiology of type 1 diabetes

A broad global variation in diabetes development exists both between and within countries. The overall age adjusted incidence of type 1 diabetes ranges between 0.1 per 100,000 per year in Venezuela to 36.8 per 100,000 per year in Sardinia (2) and

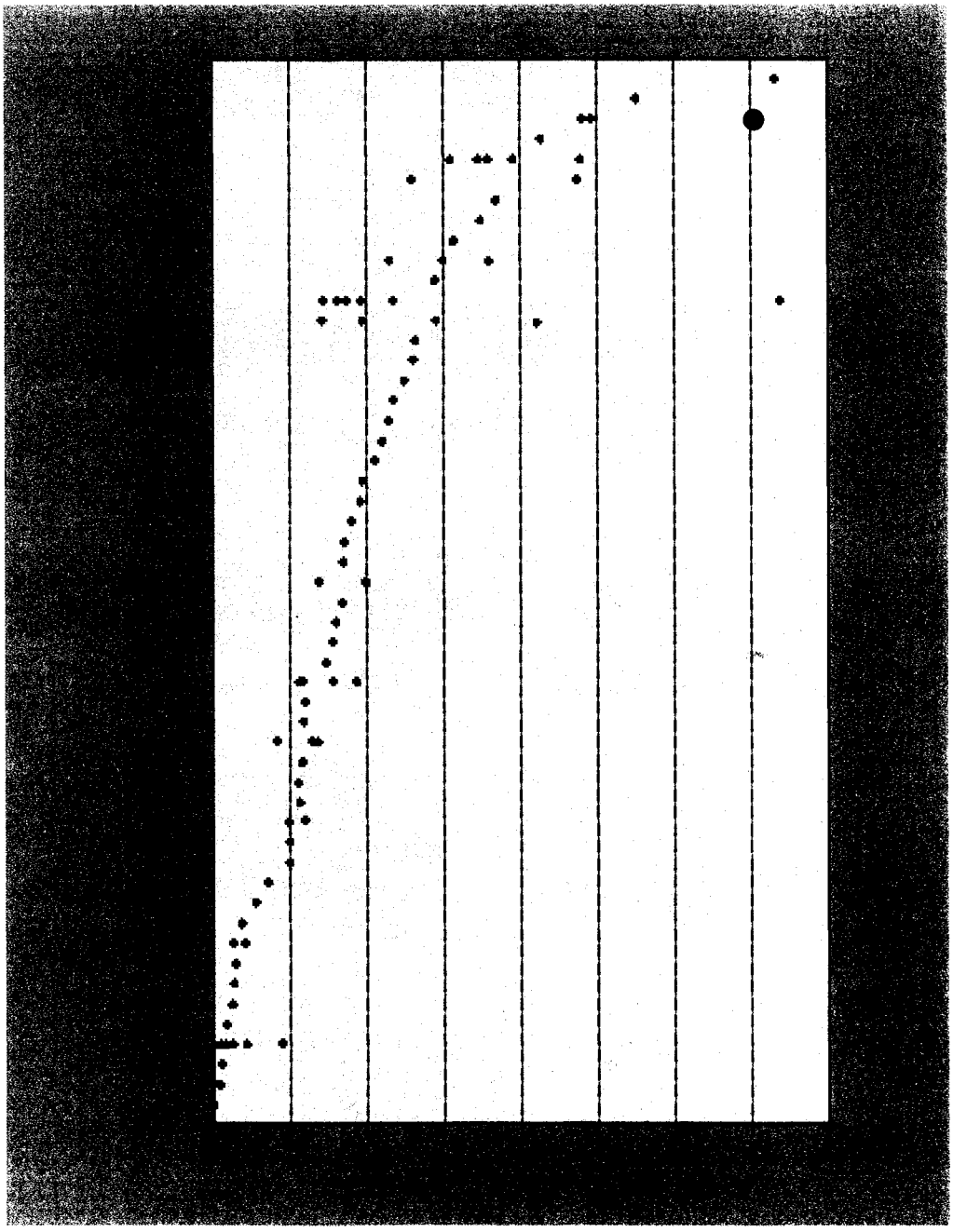
Figure 1. Integrative biology of type 1 diabetes. There may be several diabetes genotypes, each of which may interact with one or more environmental factors. The exact event or combination of circumstances that initiates the immune attack on the β cells is not known. As the process wears on, the normal repair processes cannot counteract the loss of β cells, until finally glucose homeostasis can no longer be maintained. Attempts to prevent or reverse the process have focused mostly on various forms of immunosuppression, islet transplantation or avoidance of potential environmental diabetogens. The complexity of the interactions may be better understood as newer technologies reveal diabetes-related patterns in the target tissue, effector cell populations and as the identity of various environmental diabetogens becomes known. MHC, major histocompatibility complex; Th, T helper lymphocyte. Modified and reprinted with permission from the chapter Environmental agents and type 1 diabetes, in *Textbook of Diabetes, third edition*, edited by J.C. Pickup and G. Williams, pp. 17.1-17.6 published in Oxford by Blackwell Sciences Ltd. (2003).


Genes
Environment
Diet
Virus
Toxins/
Stress
↑ Th1/Th2 Cells
Islet Mass ↓
MHC I ↑
β-Cell Death
Repair/
Immune
Counter-regulation
Type 1 Diabetes

37.5 per 100,000 in Finland (3) (Figure 2). The Avalon Peninsula in Newfoundland has a diabetes incidence of 36 per 100,000, not only the highest incidence in Canada (and North America), but also amongst the highest in the world (4). The difference represents more than a 350 fold variation in disease incidence among 100 populations worldwide. Even among genetically similar ethnic groups, large variations of disease incidence are observed. For instance, the EURODIAB TIGER study found that a more than 10 fold difference in disease incidence existed among the European nations (5). Incidence rates are highest in Northern and North western Europe in comparison with those in Central, Southern and Eastern Europe, with Sardinia being an exception (5). The large variation cannot be explained by genetic differences, as European populations are relatively homogeneous in comparison with indigenous populations from other continents (6), implicating environment as a key player in the development of diabetes.

The prevalence of type 1 diabetes is increasing worldwide. The global increase in diabetes incidence from 1960 to 1996, as determined from 37 populations, was approximately 3% annually in children between the ages of 0 and 14 years (7). It is estimated that the incidence of T1D in the year 2010 could be as high as 50 per 100,000 in Finland (which may have already occurred) and 30 per 100,000 in many other countries (7). This rate of increase is too high to be attributed to changes in the frequency of susceptibility genes (2, 8). Instead, changes in environment are likely playing a major role in the increased incidence of diabetes.

Figure 2. Geographic distribution of type 1 diabetes. Age-standardized incidence per 100,000 per year of type 1 diabetes in children ≤ 14 years of age in 100 populations. Data for boys and girls have been pooled. The incidence of diabetes in the Avalon Peninsula region of Newfoundland, Canada is indicated by the black dot. Modified and reprinted with permission from *Diabetes Care* vol. 1, pp. 1516-1526 (2000).



Genetics of type 1 diabetes

Type 1 diabetes occurs in genetically susceptible individuals possessing various combinations of risk genes, the penetrance of which are highly variable and dependent on environmental factors (9, 10). In total, over twenty loci with varying penetrance have been associated with diabetes risk. The major histocompatability complex (MHC) class II (human leukocyte antigen (HLA)) genes account for approximately 50% of the familial aggregation of the disease (11). The locus *IDDM1*, which is located on chromosome 6p21.3, contains the *HLA-DR/DQ* susceptibility genes. The DR3/DR4 heterozygous genotype synergistically confers the highest diabetes risk, followed by the DR4 and DR3 homozygous genotypes. About 30-50% of patients are DR3/DR4 heterozygotes (12). The MHC class II genes DQ8 (HLA DQA1*0301-DQB1*0302) and DQ2 (HLA DQA1*0501-DQB1*0201) are both associated with high risk for diabetes with 90% of diabetes patients carrying either DQ8 or DQ2 (12, 13). Approximately 35% of patients are heterozygous for both susceptibility loci, being DR3/DR4, DQ2/DQ8 in comparison to only 2% of the general population (13).

The *IDDM2* diabetes susceptibility locus another major contributor to risk and is located on chromosome 11p15. Susceptibility is dependent on a variable number of tandem repeats located about 0.5 kb 5′ of the insulin gene, *INS* (14-17). A short set of repeats (26-63 repeats) confers low insulin expression and correlates with a high risk of diabetes (17). Diabetes risk decreases with increases in the number of repeats (18-20).

Other risk genes include manganese superoxide dismutase (21), cytotoxic lymphocyte antigen-4 (22), the MHC-class I related gene A, *MICA* **(23)**, as well as insulin-like growth factors (24-26). It is likely that even more susceptibility loci will be

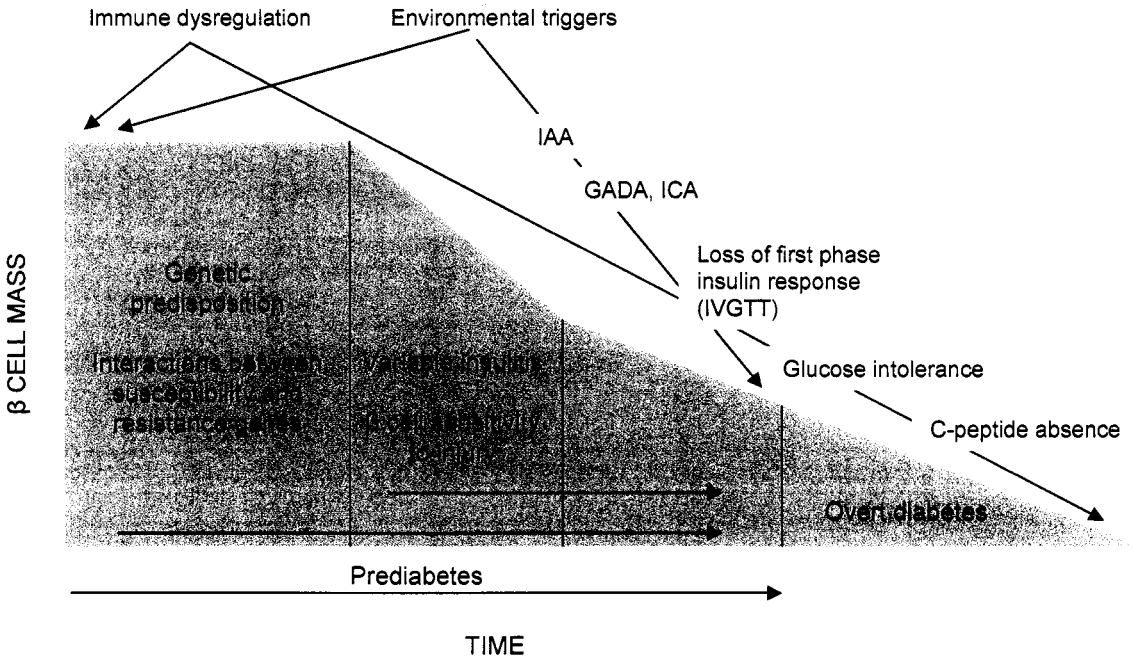
identified, as current information about risk genotypes is incomplete. Recent genome wide scans have identified new susceptibility regions that will require finer mapping to determine the specific genes involved (27-29). Also, screening single nucleotide polymorphisms in known risk genes will allow for the identification of specific alterations in gene products, which in turn can lead to the elucidation of mechanisms that result in susceptibility (30-32). In the end, the task to identify the full complement of risk genes will likely prove difficult as the effect of most risk genes is weak, with the exception of HLA region susceptibility genes (10). Many risk genes act synergistically and under the influence of environmental factors to cause diabetes development (33-35).

Pathogenesis of autoimmune diabetes

Type 1 diabetes is an autoimmune disease that results when a chronic inflammatory process of unknown origin destroys most of the insulin-producing β-cells in the pancreatic islets of Langerhans (Figure 3) (36). In a healthy individual, autoreactive T cells are kept in check by a variety of regulatory mechanisms (reviewed in (37)). Most autoreactive T cells are deleted during central T cell selection in the thymus. Normally, autoreactive T cells that escape thymus selection stay in check by remaining ignorant of autoantigen, by peripheral deletion, or by regulation that results in anergy, inhibition or suppression. Therefore, a state of self tolerance is achieved and no damage to self tissues occurs.

The progression of T1D begins when β-cell reactive mononuclear cells are activated by an unknown mechanism and infiltrate the islets. This T helper 1 (Th1)-biased, pro-inflammatory state in which mononuclear cells infiltrate the islets is termed

Figure 3. The natural course of diabetes development. In this model of diabetes development, β-cell mass changes with age. The chronic interaction of various genes, immune dysregulation and environmental triggers and regulators result in a nearly complete loss of functioning β cells, glucose intolerance and finally overt diabetes. Clinical manifestations of diabetes include the development of autoantibodies, the loss of first phase insulin response, glucose intolerance and the loss of the serological marker C peptide. GADA, glutamic acid decarboxylase antibodies; IAA, insulin autoantibody; ICA, islet-cell antigen; IVGTT, intravenous glucose tolerance test. Modified and reprinted with permission from *The Lancet* vol. 358, issue 9277, pp. 221-229 (2001).


Immune dysregulation
Environmental triggers
IAA
GADA, ICA
Loss of first phase insulin response (IVGTT)
Glucose intolerance
C-peptide absence
Genetic predisposition
Overt diabetes
β CELL MASS
Prediabetes
TIME

insulitis. During the early stages of insulitis, macrophages and dendritic cells (DCs) enter the islets, followed by $CD4^+$ and $CD8^+$ T lymphocytes, natural killer (NK) cells and B cells (38, 39). Macrophages and DCs are assumed to play a key role in disease induction as they are professional antigen presenting cells (APCs) that can present autoantigens to inflammatory $CD4^+$ helper T cells in the context of MHC class II-peptide complexes. The activated T cells proliferate by clonal expansion and elicit β-cell specific immunity and nonspecific inflammatory responses (1). Once insulitis begins, it can become a self-perpetuating cycle of inflammation. Antigen spreading occurs as β-cell death exposes sequestered self antigens, providing more material for stimulation of the autoreactive lymphocytes and other immune cells (40). The presence of islet cell autoantibodies, cellular immune reactivity to islet cell antigens and lymphocytic infiltration in the islets supports the classification of T1D as an autoimmune disease (1).

Diabetes can remain subclinical for months to years. The development of autoantibodies to diabetes-related autoantigens, such as insulin, glutamic acid decarboxylase (GAD) and the protein tyrosine phosphatase-like IA-2/IA-2β (IA-2), acts as a marker of islet autoimmunity and disease progression (Figure 3). The loss of first phase insulin response (IVGTT) in individuals is an indicator of reduced β-cell function. The consequence of decreased β cell mass is a reduction in endogenous insulin production resulting in clinical manifestations and the onset of overt diabetes. Clinical symptoms include polyuria, weight loss, fatigue, hyperglycemia and ketoacidosis. Overt diabetes is characterized by glucose intolerance and the loss of the serological marker C-peptide (1, 41). The patient must begin a life long regime of daily glucose monitoring and

injection of exogenous insulin to maintain glucose homeostasis. Without insulin treatment, patients rapidly develop ketoacidosis, lapse into a diabetic coma and die.

Markers of islet autoimmunity

Although type 1 diabetes is thought to be an inflammatory T cell-mediated disease, it is the presence of autoantibodies that marks the progression of the autoimmune process in humans and animals. Studies of the prediction and pathogenesis of T1D in humans rely on serum autoantibodies as biomarkers of the destructive process. The fact that the appearance of autoantibodies correlates with histologic damage in the pancreas of newly diagnosed patients was confirmed in a unique study of tissue obtained by pancreatic biopsy (42). The most commonly used markers in human studies are autoantibodies against proinsulin and insulin, GAD, and IA-2 (1, 40, 41).

All of the major autoantigens associated with T1D were identified using antibodies isolated from diabetic individuals. The first diabetes-related autoantigen was identified as a 64 kDa protein that was bound by antibodies from diabetic children, diabetes-prone BioBreeding (BBdp) rats and non-obese diabetic (NOD) mice (43-45). The 64 kDa autoantigen was subsequently identified in patients concordant for both the neurologic disease, Stiff-man syndrome and type 1 diabetes, as GAD (46). GAD is considered to be one of the key autoantigens in type 1 diabetes.

Animal models of type 1 diabetes

There are two major animal models of spontaneous polygenic autoimmune diabetes, the diabetes-prone BioBreeding rat and the non-obese diabetic mouse.

Diabetes-prone BioBreeding rat. The BBdp rat strain was discovered in 1974 in a colony of outbred Wistar rats at the BioBreeding Laboratories in Ottawa, Ontario, Canada (47). Selective breeding has achieved a cumulative diabetes incidence as high as 90% in some colonies (48). The cumulative diabetes incidence for animals fed a standard cereal-based diet is 65% in the colony housed at the Health Canada Animal Resources Division, Ottawa, Ontario, Canada (49). A parallel strain of diabetes-resistant BB control (BBc) rats that do not spontaneously develop diabetes was derived from an early subline of animals from the original BB rat colony in Ottawa.

The disease phenotype of the BBdp rat closely resembles that of humans (48). Clinical diabetes appears during adolescence between 60 and 100 days of age and is sex-independent (50). Insulin response declines as β-cell mass declines (51) and clinical onset of disease occurs in a rapid fashion manifesting as hyperglycemia accompanied by weight loss, hypoinsulinemia, ketonuria and ketoacidosis (48). As in humans, mononuclear cell infiltration occurs within and around the islets of Langerhans (47, 50). Insulitis occurs two to three weeks before overt diabetes and leads to the complete selective destruction of β-cells. The lymphoid cells that are found in islet lesions include macrophages and DCs followed by $CD4^+$ and $CD8^+$ T cells, NK cells and B cells (48). The cytokine profile associated with insulitis is inflammatory and includes interferon-γ (IFN-γ), tumor necrosis factor (TNF), interleukin-1 (IL-1), IL-12 and IL-6 (52-55). As in humans, autoantibodies to islet cell antigens appear in the serum of prediabetic BBdp rats

(50).

BBdp rats share some of the major risk genes for diabetes with their human counterparts. All BBdp rats share the risk gene *iddm2* which is associated with the MHC locus on chromosome 20 (50). Diabetes expression requires the MHC class II $RT1^u$ allele (56). BBdp rats carry a mutation at the *iddm1/lyp* locus on chromosome 4 that results in T cell lymphopenia (56-58). This gene has been identified as *Ian5*, a novel member of the immune-associated nucleotide (IAN)-related gene family. Lymphopenia is due to a frameshift deletion, resulting in truncation of a significant portion of the protein that could be important in T-cell development (58).

Non-obese diabetic mouse. The NOD mouse strain originated from the Shionogi Aburahi Laboratories in Japan (59). It was derived from a line of CTS mice characterized by cataracts and small eyes. Cumulative diabetes incidence varies among colonies but is usually approximately 90% in female mice (60, 61). The diabetes-resistant mouse strains, NON and NOR, were developed in parallel with the NOD mouse strain.

The NOD mouse develops autoimmune T1D spontaneously. Mononuclear cell infiltration into the islets can be observed as early as three weeks of age. Insulitis accumulates *en masse* around the islets (peri-insulitis) with β-cells remaining intact until approximately 15 weeks of age. At this point, destruction of the β-cells is rapid, followed by the development of overt diabetes (62). Nearly all male and female NOD mice develop insulitis by 20 weeks of age (62). However, the development of overt diabetes is sex-dependent with the majority of affected animals being female. The mononuclear cells that infiltrate the islets are predominantly $CD4^+$ and $CD8^+$ T cells, but also include macrophages, DCs and B cells (62). The clinical symptoms observed in diabetic NOD

mice are similar to those observed in humans, including polyuria, polydipsia, weight loss and dependence on exogenous insulin (58).

The major susceptibility genes in NOD mice are similar to those in humans and BBdp rats. One of the major susceptibility loci is *Idd1* in the MHC region on chromosome 17 (63-66). Interestingly, the NOD MHC class II has been shown to be essential but not sufficient for the development of diabetes (67-70).

Environmental agents and diabetes pathogenesis

Although genetic susceptibility is required to develop type 1 diabetes, it is not usually sufficient alone to produce diabetes (71-73). Environmental factors including certain viruses and dietary components have been implicated in the initiation of the autoimmune process. Aside from the epidemiological data, twin studies also support the hypothesis that, in many cases, environmental agents are essential for disease development (71-73).

Studies of the environmental contribution to the development of type 1 diabetes have focused on disease concordance between monozygotic twin pairs. If diabetes development were dependent on genetic predisposition alone, then both individuals from a genetically identical, monozygotic twin pair would develop disease. Indeed, as expected, monozygotic twins have an increased risk of diabetes compared with dizygotic twins and non-twin siblings (71, 73). However, the concordance rate among monozygotic twins is not 100%, indicating that environment contributes to diabetes induction and pathogenesis. The concordance rate among monozygotic twins is between 30-50% and is dependent on the age at diagnosis of the proband (71-75). When the proband twin is

diagnosed before 5 years of age, the concordance rate is 65-70% (72, 73). Concordance rates in twin pairs in which initial diagnosis is after 15 years of age range from 18-38% (71, 74, 75).

Interestingly, among monozygotic twin pairs that are discordant for diabetes, β-cell autoimmunity often occurs in the non-diabetic twin. In discordant monozygotic twin pairs, 20 to 66% of the non-diabetic twins developed autoantibodies to IA-2, insulin and/or GAD, indicating the presence of an autoimmune attack and damage to β-cells (75, 76). These observations suggest that there is genetic predisposition to develop an autoimmune process against islet cell antigens, but that environmental stimuli play a role in the progression to overt diabetes.

What are the candidate environmental players?

Although it is well accepted that environmental agents promote the development of type 1 diabetes, the identification of specific perpetrators remains elusive (77). It has been difficult to identify candidate diabetes-related environmental agents because of the multifactorial nature of the disease (36, 77). Linking past exposures to the later development of diabetes, the lack of knowledge of the pathogenic mechanism of offending environmental stimuli, and the large number of predisposing genes and the number of different risk genotypes, all complicate the search for environmental agents involved in diabetes development (10). To date, the two most studied environmental factors are viruses and diet.

Virus-mediated diabetes. Viruses are thought to target and destroy β-cells by at least two mechanisms. It has been shown that some viruses can be directly cytotoxic to β-

cells, while others can indirectly trigger the β-cell specific autoimmune process (78). Viruses, such as Rubella, mumps, cytomegalovirus (CMV), enteroviruses (EVs) and retroviruses have all been directly or indirectly linked to diabetes development.

Rubella is the only virus that has been strongly linked to diabetes. Within 5 to 25 years after infection, 10 to 20% of patients diagnosed with congenital rubella syndrome develop autoimmune diabetes (79-82). However, the link between other viruses and diabetes is more tenuous. Temporal relationships have been observed between mumps or CMV infection and autoantibody development and/or onset of diabetes (83-85). Retroviruses have been implicated by the cross-reactivity between the retroviral antigen p73 and insulin autoantibodies from T1D patients (86). Contrasting observations linking these viruses and diabetes development (87) indicate that they may only be involved in a small subset of individuals.

Enteroviruses have been implicated in the development of autoimmune diabetes (87, 88) but a specific diabetes-inducing enterovirus is yet to be identified (89). EVs belong to the picornavirus family, a group of RNA viruses that typically infect the stomach and intestine (90). EVs were initially linked to T1D through a temporal association between coxsackie B virus (CBV) infection and disease incidence (91-97). CBV induced diabetes in mice is associated with an induction of autoantibodies against GAD (98). Also, GAD autoantibodies have been shown to be cross-reactive with a 6-residue homologous motif, PEVKEK, in the CBV-P2 protein (99, 100). The identification of this cross-reactive peptide suggests the possibility that CBV can induce diabetes via molecular mimicry. It is thought that EVs in general could trigger or potentiate existing β-cell autoimmunity either by direct cytotoxicity or by indirect effects

on cells of the immune system (101, 102).

Despite the interest in virus-mediated diabetes, epidemiological evidence of infectious hotspots or traceable routes of infection is lacking (103, 104), and there are conflicting data with respect to the presence of candidate viruses in the pancreas or immune cells of diabetic patients (105-107). Also, spontaneous diabetes development still occurs in BB rats and NOD mice maintained under gnotobiotic or axenic conditions (108) [Scott *et al*, unpublished data]. Because animals maintained in strict viral antibody-free and germ-free conditions still develop diabetes, diet remains the major environmental stimulus.

Dietary modification of autoimmune diabetes. A number of dietary factors have been associated with diabetes development. These include milk proteins, wheat proteins, vitamin D and *N*-nitroso compounds. The discussion in this paper will focus on milk and wheat proteins and their relationship to type 1 diabetes.

Cow milk proteins and their effect on islet autoimmunity has been studied intensely with the publication of more than 25 case-control studies examining the link between early exposure to cow milk, lack of breast-feeding and later development of T1D having been published. Cow milk exposure early in life has been linked to the development of diabetes in humans (109-111), diabetes-prone BB rats (111, 112) and NOD mice (113, 114). However, the data have been contradictory with some studies showing a relationship and others not (115, 116). It was calculated that humans exposed to cow milk early in life had an odds ratio of approximately 1.6 of developing diabetes (109, 117). However, the TRIGR trial, a prospective nutritional intervention trial in humans, showed that genetically high risk infants fed a hydrolyzed casein (HC)-based

infant formula were less likely to develop islet cell autoantibodies (118). It could be that milk-based diets induce diabetes in a subset of susceptible individuals but the diabetes-inducing potential varies, likely due to the genetic variation in cow proteins (119).

Milk protein was observed to induce diabetes in NOD mice and it was hypothesized to be attributable to the A^1 β-casein variant rather than the A^2 variant (120). Similarly, an Icelandic group reported that β-casein fractions can influence type 1 diabetes incidence in patients (121). In contrast, a recent international trial, in which standardized diets containing purified milk β-casein variants A^1 or A^2 were fed to NOD mice and BB rats, showed that in most instances diets based on either variant protected the animals from developing diabetes (122). In fact, the same study demonstrated that a milk-free, wheat-based diet produced the highest diabetes frequency in BBdp rats and NOD mice in three widely separate locations (122). Thus, the controversy surrounding milk may be due to a small subset of milk-sensitive, at-risk individuals distributed within the total at-risk population and is further complicated by variable milk composition.

Wheat contains a complex mixture of proteins. Prolamin is a general term for cereal storage proteins in the plant endosperm and they comprise 80% of the total wheat protein. Wheat flour consists predominantly of wheat gluten proteins (gliadins and glutenins), but 15% of the protein is made up of soluble albumins and globulins (123). Specific wheat proteins are known to cause inappropriate immune stimulation in susceptible individuals. For example, celiac toxic gliadin peptides cause immune-mediated damage to the gut (124, 125) and water and salt soluble wheat allergens are involved in IgE mediated Baker's asthma (126).

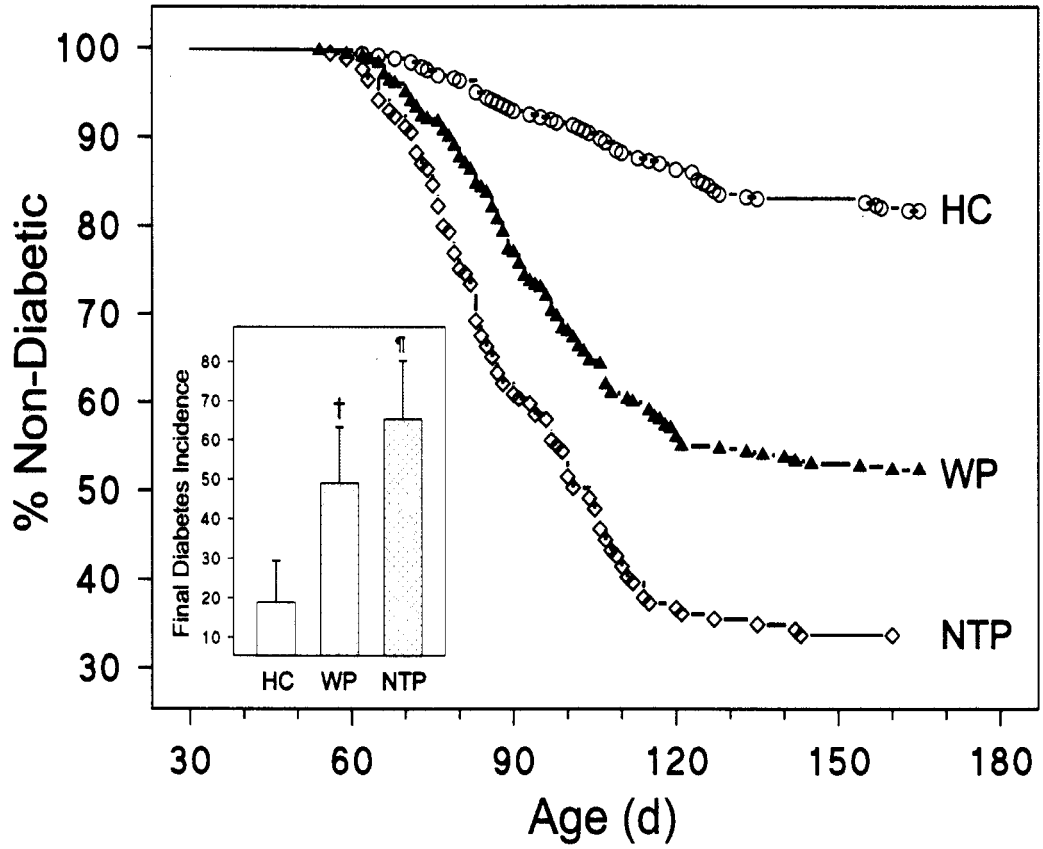
Animal studies have shown that cereal-based diets are associated with the onset of

diabetes (116, 127). The incidence of disease in BBdp rats fed a standard rodent chow diet (NTP-2000 diet, NTP), in which the main protein source is wheat, is approximately 65% (Figure 4) (101, 116, 127). When rats are fed a protective HC diet, the incidence of disease is approximately 20%, while a wheat protein (WP) diet increases it to 50% (49, 116). The sole amino acid sources in these two semipurified isocaloric isonitrogenous diets are HC and WP. Our lab characterized the diabetes-inducing potential of all the major ingredients of the NIH-07 diet (mixed, cereal-based diet), which is composed of 83.5% plant materials and found that wheat gluten (WG) was the major diabetes-inducing component (116, 119). A wheat-based diet also influences the degree of insulitis in BBdp rats and can modify cytokine production by enterocytes or immunocytes, potentially lowering the threshold of the immune response by enhancing maturation of APCs (128, 129). Wheat-based diets have also been associated with the induction of diabetes in NOD mice (114, 130-132). These data are consistent with the involvement of dietary wheat proteins in diabetes pathogenesis (116, 127, 133). However, the identity of the specific diabetogenic agents in wheat and the mechanisms by which they participate in diabetogenesis remain unclear.

Celiac disease: A model of dietary wheat immunomodulation

Parallels can be drawn between the pathogenic effect of wheat in the wheat sensitive enteropathy, celiac disease (CD) and its effect in diabetes. CD is a wheat-induced immune-mediated disease caused by the exposure of susceptible individuals to storage proteins from wheat, barley, and rye. This food-induced immune disorder involves an inflammatory, IFN-γ-dependent immune response to gluten protein which

Figure 4. Modification of diabetes development in diabetes-prone BB rats by wheat-based diets. Survival curves and final diabetes incidence (*inset*) in BBdp rats fed from weaning a mixed, cereal-based (mainly wheat) NTP diet or two semipurified, isocaloric, isonitrogenous diets in which the sole amino acid source was either hydrolyzed casein (HC) or wheat gluten protein (WP) plus supplemental sulfur amino acids. The rate of diabetes development was significantly delayed in rats fed the HC diet in comparison to both NTP- and WG-fed rats (log-rank/Kaplan-Meier test). *Inset*, animals fed the NTP diet had the highest incidence, 65 ± 15% (x ± SD, $n = 6$ experiments, total of 169 rats). There were more cases of diabetes in BBdp rats fed WP diets ($n = 12$ experiments, total of 282 rats, 51 ± 11%) than those fed a protective HC diet ($n = 14$ experiments, total of 322 rats, 19 ± 11%, 14 experiments). ANOVA/Scheffé's; ¶ denotes $p = 10^{-6}$ vs. HC; † denotes $p = 10^{-5}$ vs. HC. Modified and reprinted with permission from *Journal of Biochemistry* vol. 278, pp. 54-63 (2003).


% Non-Diabetic
100
90
80
70
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50
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30
30
60
90
120
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Age (d)
HC
WP
NTP
Final Diabetes Incidence
80
70
60
50
40
30
20
10
†
¶
HC
WP
NTP

produces intestinal damage (134).

Celiac disease or celiac-like symptoms are often found to be associated with the development of diabetes. Two to six percent of diabetic patients have overt celiac disease, an incidence six to 20 times that found in the general population (135-139). Interestingly, diabetes develops first in 90% of these cases (133). The implication is that with the diagnosis of CD and the subsequent removal of wheat protein from the diet could inhibit the further development of diabetes. The two diseases have certain common risk genes, in particular the HLA-DQA1*0501/DQB1*0201 allele (DQ2), which may explain some but not all cases of coincident CD and diabetes (135, 140, 141). Diabetic patients who do not have biopsy proven CD still demonstrate high levels of antibodies to tissue transglutaminase (tTG), the CD-associated autoantigen (142). In a group of 68 type 1 diabetic patients who were DQ2 homozygotes, 33% had IgA antibodies to tTG (143). Another report indicates that 10% of patients have tTG antibody levels similar to those of CD patients and another 30% had low level tTG antibody binding (144). Furthermore, newly diagnosed diabetic infants presented with anti-gliadin antibodies (145, 146). The observed association between celiac disease and diabetes is consistent with the idea that immunity to wheat plays a role in the pathogenesis of diabetes in a subset of susceptible individuals.

A case for dietary wheat and its involvement in the development of diabetes

Aside from the coincidence of diabetes and celiac disease, there are a growing number of studies that associate a CD-independent wheat-modification of diabetes development. As previously mentioned, both rodent models of spontaneous diabetes

develop diabetes at an increased frequency when fed a diet containing cereal proteins. Two large independent prospective studies of children genetically at-risk of developing diabetes indicate that early oral exposure to cereal proteins increased the risk for islet autoimmunity. The DAISY study in Denver, Colorado found that at-risk children exposed to cereals earlier than 3 months or later than 7 months of age had increased risk of islet autoimmunity (147). The BABYDIAB study in Munich, Germany was more specific in their analysis of food supplementation, breaking cereals into gluten containing or non-gluten containing groups. They found that food supplementation with gluten containing cereals before 3 months of age was associated with increased risk of islet autoimmunity (148). The data suggest that not only the introduction of (gluten containing) cereals, but also the timing of the introduction can affect diabetes outcome, as indicated by the presence of islet autoantibodies.

The effect of eliminating gluten from the diet of at-risk individuals was recently tested. Wheat gluten was removed from the diet in an attempt to decrease islet autoimmunity in first-degree relatives of T1D patients who had autoantibodies to at least two autoantigens. Although the diet change did not alter the autoantibody status of the patients, it did increase their first phase insulin response to glucose, a measure of β-cell mass (149). Therefore, the removal of wheat proteins from the diet cannot stop an ongoing autoimmune process, but it can positively affect β-cell mass and/or function. Thus, it will be of interest to identify wheat-sensitive individuals at risk for T1D in whom it could be possible to prevent the development of islet autoimmunity or prolong the prediabetes period by enhancing glucose tolerance by removal of gluten from the diet.

Diabetic individuals have demonstrated wheat-specific cellular immunity that is

independent of celiac disease. It has been observed that wheat proteins can stimulate the immune system in BBdp rats and NOD mice (128, 150, 151). Bone marrow-derived DC from mice were induced to mature upon exposure to WG, as determined by the expression of the surface markers MHC class II, CD40, CD54, and CD86 (129). Wheat gluten also induced the secretion of the chemokines MIP-2 and keratinocyte-derived cytokine. Therefore, it was proposed that wheat gluten could, in genetically susceptible individuals, lower the threshold for immune responses by causing maturation of APC, by attracting leukocytes and increasing their reactivity state (129). In support of this hypothesis, our lab has demonstrated that mixed lymphocyte populations from BBdp rats can be stimulated to proliferate by wheat gluten (Scott *et al*, unpublished data). Also, T1D patients have displayed increased T cell stimulation in response to wheat gluten in comparison with control subjects (152) (Scott *et al*, unpublished data). Furthermore, 20% of diabetic children with no clinical signs of CD showed activation of rectal (*in vivo*) and jejunal (*in vitro*) lamina propria and epithelium $CD3^+$ and $\gamma\delta^+$ lymphocytes in response to gliadin (153, 154). The data support the hypothesis that a subset of diabetic patients is susceptible to immune modification and activation by wheat and that this effect can be found independent of the development of the wheat sensitive enteropathy, celiac disease.

The knowledge of the offending environmental antigen involved in the development of celiac disease has allowed for the clarification of the pathogenic mechanism. Specific aspects of CD have now been elucidated, including the identification of gliadin B and T cell epitopes, how gliadin is presented to the immune system by MHC class II molecules on APCs, the role of tTG as an autoantigen, and the details of the physiological outcomes such as intestinal inflammation. Until a specific

offending environmental antigen is found to be associated with autoimmune diabetes, the pathogenic mechanisms will remain elusive, thwarting the development of preventive and therapeutic measures for susceptible individuals. The main aim of the present work was to identify and characterize diabetes-related wheat proteins in three separate species, rat, mouse and human.

HYPOTHESIS

The hypothesis is that specific wheat proteins induce diabetes in a subset of genetically susceptible individuals. These wheat proteins can act via a number of mechanisms, including molecular mimicry, the alteration of cytokine profiles in pathogenically important tissues, or the modification of the target tissue. The immune reaction to specific wheat proteins results in antibody production. In turn, these antibodies can be used to identify the specific diabetes-promoting wheat proteins.

EXPERIMENTAL QUESTIONS

Research question 1: What specific wheat proteins are involved in the development of type 1 diabetes?

Aim and approach: To identify diabetes-related wheat proteins by immunoscreening a wheat cDNA expression library with pooled immunoglobulin G (IgG) serum antibodies from diabetic BB rats.

Rationale: Despite continued progress in the elucidation of diabetes pathogenesis, the environmental antigens that initiate and maintain the process leading to β-cell destruction

remain unknown. It was hypothesized that BBdp rats fed a wheat-based diet develop antibodies to unique, diabetes-related immunoreactive wheat proteins. Serum antibodies have been used by other groups to identify candidate antigens involved in diabetes development, such as viral envelope proteins (86) and GAD (43, 46). A similar approach has also been used to identify various environmental allergens, such as grass pollen and peanut allergens (155-157). Therefore, we proposed to take a similar approach and use IgG antibodies to identify diabetes-related wheat proteins.

Research question 2: Do individual diabetic BB rats, in comparison with asymptomatic BBdp or BBc rats, have increased antibody reactivity to the identified wheat proteins and does this antibody reactivity correlate to damage in the target tissue?

Aim and approach: To determine whether the wheat proteins isolated from the wheat cDNA library are associated with target tissue damage and disease development in the BBdp rat. In order to achieve this aim, the candidate wheat proteins were screened with IgG antibodies from individual diabetic, asymptomatic and control animals. The proteins were deemed diabetes-related if antibody reactivity and frequency of antibody development was associated with diabetes status. Also, insulitis scores and the percent of infiltrated islets were assessed for the individual BB rats studied. It was determined whether a correlation existed between antibody reactivity to wheat proteins and damage to the target tissue, further supporting their role in diabetes development.

Rationale: Screening the library with pooled antibodies from diabetic individuals allowed for the identification of candidate diabetes-related wheat proteins. However, it was necessary to determine whether there was a disease-state dependent correlation with

antibody production. A correlation between diabetes status and antibody reactivity would support the identity of the wheat proteins as diabetes-related antigens.

In T1D patients, the presence of autoantibodies to either GAD or IA-2 has been shown to be closely correlated with pancreatic islet inflammation (insulitis) and hyperexpression of MHC class I antigens in islets (42). Therefore, a correlation between antibody reactivity to specific wheat proteins and islet inflammation and insulitis score in animals at different risk for diabetes could represent an association between dietary wheat antigens and progression of disease, further supporting a link between specific dietary antigens, islet autoimmunity and diabetes.

Research question 3: Is diabetes in mice and humans also associated with increased antibody reactivity to the candidate wheat proteins?

Aim and approach: To confirm that increased antibody reactivity to the identified diabetes-related wheat proteins is associated with the development of spontaneous autoimmune diabetes by demonstrating it in two other species, namely mice and humans. Therefore, the candidate proteins will be immunoscreened with serum antibodies from (pre)diabetic NOD and diabetes-resistant NOR mice, as well as from T1D patients and healthy controls.

Rationale: By immunoscreening the candidate proteins with antibodies from prediabetic or diabetic individuals and healthy controls, we can determine whether they are also associated with diabetes development in two other species, confirming the observations previously made in BBdp rats.

Research question 4: Do diabetes-related wheat proteins share sequence and structural homology with known immunomodulatory or self-proteins?

Aim and approach: To determine whether the identified wheat proteins share homology with autoantigens, infectious agents, or immune system structures involved in diabetogenesis. The identified diabetes-related wheat proteins were analyzed for DNA and amino acid sequence homologies with known diabetes-related antigens, immunomodulatory proteins and proteins from other plant species. General searches of the Genbank database and of the human genome were also performed to identify novel homologous proteins or peptides for which the wheat proteins may act as molecular mimics. To identify antigenic epitopes, a B cell epitope library of the candidate diabetes-related wheat protein was screened with IgG antibodies from a highly wheat sensitive patient with both T1D and CD. Also, MHC class II peptides for the candidate wheat proteins were predicted.

Rationale: Molecular mimicry has been proposed as one of the mechanisms responsible for the development of type 1 diabetes (158). Homologies between the wheat proteins and immunomodulatory, self or target tissue antigens could help elucidate the role of wheat proteins in diabetes development.

MATERIALS AND METHODS

Patients

Case study. The case of a 28 year-old Caucasian female with both type 1 diabetes and celiac disease was studied. The patient was found to have the shared diabetes and celiac-associated HLA haplotypes DRB1*03/*04 and DQB1*0201/*0302 (DQ2/DQ8). Control subjects with no significant medical history and with an age range of 22-40 (n = 8; mean age 29 ± 6.8 years; 6 female, 2 male) were included.

Patient study group. Patients and healthy control subjects were recruited in accordance with the guidelines set out by the Ottawa Hospital Research Ethics Board and the Children's Hospital of Eastern Ontario Ethics Board. Permission for blood sampling was given by study subjects with informed consent. Diabetic patients had an age range of 8 to 38 years (n = 22; mean age 22.7 ± 7.1 years; 12 females, 10 males). Celiac disease patients had an age range of 25 to 33 years (n = 3; mean age 28.3 ± 4.2; 2 females, 1 male). Patients with both type 1 diabetes and celiac disease had an age range of 12 to 43 years (n = 3; mean age 27.7 ± 15.5 years; 3 females). Control subjects with no significant medical history and with an age range of 21 to 40 (n = 14; mean age 27.2 ± 5.7 years; 9 females, 5 males) were included.

Blood samples were taken for serum and PBMC isolation. Serum was tested for IgA antibodies to the celiac-associated autoantigen tissue transglutaminase. PBMC from patients and control subjects were isolated from blood using Histopaque-1077 (Sigma Diagnostics). DNA HLA typing for DRB1 and DQB1 molecules was performed on genomic DNA extracted from the patient and control subject PBMC. These tests were

performed by the Ottawa Hospital; General Campus Biochemistry Lab. PBMC isolation was performed by Majid Mojibian, David Lefebvre and Habiba Chakir in the Scott lab.

Diabetes-prone BioBreeding rats

Male and female BBdp and BBc rats were obtained from the colonies maintained at the Animal Resources Division, Health Canada, Ottawa, ON. The animals were maintainedat at 18 to 26°C in laminar flow protected cages under specific pathogen-free conditions. The light-dark cycle (light:dark 12:12) with lights on from 9 a.m. to 9 p.m. Tests in sentinel animals indicate the colony is antibody-free with respect to Sendai virus, pneumonia virus of mice, rat corona virus/sialodacryoadenitis virus, Kilham rat virus, Toolan's H-1 virus, reovirus type 3, and *Mycoplasma pulmonis*.

The mean incidence of diabetes in BBdp rats from this colony fed a standard cereal-based diet (159) has remained constant at 65 ± 2% (mean ± SD). This colony is directly descended from the original diabetic rat strain discovered in Ottawa in 1974 at the BioBreeding laboratories. The colony was transferred to Health Canada in 1977. The colony is not inbred but has remained closed since that time. Genotyping for selected markers indicates the animals are approximately 80% identical at the genetic level. BBdp rats carry a mutation at the *Iddm1/lyp* locus, the same as BB/W rats, which is attributed to a frameshift deletion in a member of the immune-associated nucleotide-related gene family, *Ian5* (160). A parallel diabetes-resistant strain, BB control (BBc) rats, was derived from an early subline of the original BBdp rat colony.

Animals were weaned onto a specified diet at 23 days of age, caged in banks of 30 wire-bottom cages, and given free access to food and water. The principles of

laboratory animal care as described by the Canadian Council on Animal Care were followed.

Orbital bleeds were performed at 70 days to obtain prospective serum samples. Animals were tested twice weekly for glucose in urine using Testape (Lily, Toronto, ON) after 60 days of age. Rats with a glucose value greater than 2+ were fasted overnight, and blood glucose in tail blood was measured the next morning using a glucometer. Diabetes was diagnosed when fasting blood glucose was >11.1 mM. Diabetic animals were killed within 24 h of diagnosis by exsanguination while under anesthesia with 3% halothane or isoflurane in oxygen. The blood was collected into vacutainers (BD Biosciences, Franklin Lakes, NJ) with clot activator coated walls. The blood was spun for 20 min at 1000 x *g* at 4°C (Beckman Allegra 6R Centrifuge). Serum samples were aliquotted and stored at -20°C until use. Animals were observed for diabetes development until the end of study at 150 days.

Non-obese diabetic and non-obese resistant mice

Female NOD weanling mice were obtained from Taconic Farms, Inc. (Germantown, NY). Animals were guaranteed murine pathogen free according to Taconic Health Standards (http://www.taconic.com/healthr/healthstandards.htm, accessed Sept. 1, 2004). The animals were housed at the Health Canada Animal Resources Division and were kept at 18 to 26°C in laminar flow protected cages under specific murine pathogen-free conditions. The light-dark cycle (light:dark 12:12) with lights on from 9 a.m. to 9 p.m. Animals were weaned at 23 days onto specified diets and given free access to food and water. The principles of laboratory animal care as described by the

Canadian Council on Animal Care were followed.

Orbital bleeds were performed at 80 days to obtain prospective serum samples. Animals were tested twice weekly for glucose in urine using Testape (Lily) after 75 days of age. Mice with a value greater than 2+ were fasted overnight, and blood glucose in tail blood was measured the next morning using a glucometer. Diabetes was diagnosed when fasting blood glucose was >11.1 mmol/l. Diabetic animals were killed within 24 h of diagnosis by exsanguination while under anesthesia with 3% halothane in oxygen. The blood was collected into vacutainers (BD Biosciences) with clot activator coated walls. The blood was spun for 20 min at 1000 x *g* at 4°C. The serum was aliquotted and stored at -20°C until use. Animals were observed for diabetes development until the end of study at 270 days.

Serum samples from 70 d female non-obese resistant (NOR; $n = 7$), NOD.Itgb7$^{-/-}$ ($n = 12$) and NOD.Itgb7$^{-/-}$/L-Sel$^{-/-}$ ($n = 8$) mice were generously provided by Dr. Ed Leiter (Jackson Laboratories, Bar Harbor, Maine). These mice were all weaned onto the mainly wheat-based NIH-31 (6% fat; NIH) diet at the Jackson Laboratories.

Diets

NTP-2000 diet. The NTP diet (Zeigler Bros., Gardners, PA) is an open formula (the percentage composition is known), nonpurified diet for rodents developed by the United States National Toxicology Program of the NIEHS of the National Institutes of Health. NTP does not contain any milk protein. This is a mainly plant-based diet with wheat as the major component (37%), followed by corn, soybean meal, alfalfa meal, oat hulls, fish meal, and cellulose. The diet contains 14.6% protein, 8.2% fat, 9.9% crude

fiber, 52% carbohydrate, 10.7% moisture; the remainder is native and added micronutrients. The NTP diet used in these studies was irradiated and contained low levels of chemical and microbial contaminants (159).

Wheat protein diet. The WP semipurified diet consists of 22.5% wheat gluten (ICN Biochemicals, Cleveland, OH), 50.2% corn starch, 12.0% sucrose, 5.0% corn oil, 5.0% fiber (Solka-Floc), 3.5% AIN-76 (or AIN-93G) mineral mix (ICN), 1.0% AIN-76A (or AIN-93G), vitamin mix (ICN), supplemented with 0.2% choline bitartrate, 0.02% DL-methionine, 0.5% L-lysine, and 0.08% L-threonine to compensate for low sulfur amino acids in wheat proteins.

Hydrolyzed casein diet. The HC semipurified diet contains 51.0% corn starch, 12.0% sucrose, 20.0% casein hydrolyzate (pancreas S enzymatic hydrolyzate, Redstar Bioproducts, Mississauga, ON), 7.0% soybean oil, 5.0% fiber, 3.5% AIN-76 (or AIN-93G) mineral mix, 1.0% AIN-76A (or AIN-93G) vitamin mix, 0.2% choline bitartrate, and 0.3% L-cystine. Both semipurified diets are isocaloric and isonitrogenous.

NIH-31 (6% fat) diet — The NIH diet contains 18% crude protein, 6% fat, 5% crude fiber, 8% ash and 3% minerals. The ingredients, in order of composition, are ground wheat, ground corn, wheat middlings, ground oats, fish meal, dehulled soybean meal, soybean oil, corn gluten meal, dehydrated alfalfa meal, dicalcium phosphate, brewers dried yeast, calcium carbonate, menadione dimethylpyrimidinol bisulfite, salt, choline chloride, magnesium oxide, thiamin mononitrate, pyridoxine hydrochloride, cholecalciferol, vitamin A acetate, calcium pantothenate, ferrous sulfate, biotin, manganous oxide, cyanocobalamin, riboflavin, nicotinic acid, dl-alpha tocopheryl

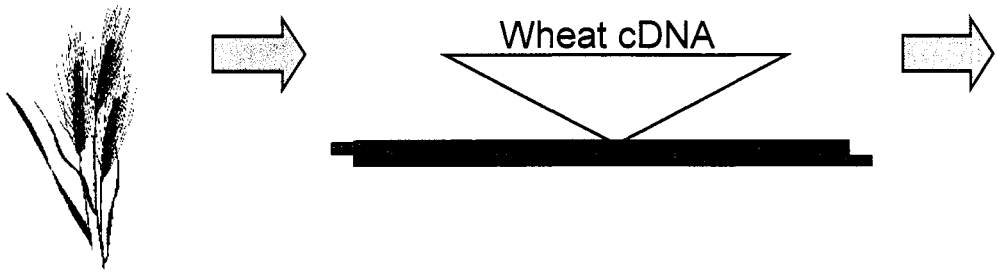
acetate, folic acid, calcium iodate, cobalt carbonate, zinc oxide, ferrous carbonate and zinc sulfate.

Wheat cDNA expression library construction and immunoscreening

Wheat cDNA library construction. (See Figure 5 for a schematic of the library screening). Total RNA was isolated from the hard red spring wheat, AC Barrie (provided by Dr. V. Burrows, Eastern Cereal Oilseed Research Centre, Agriculture and Agri-Food Canada, Ottawa, Canada). Caryopses were harvested at ~10-20 days after pollination. Total RNA was prepared by Karolina Burghardt following the method of Verwoerd *et al* (1989) (161). The RNA was sent as a pellet in absolute ethanol to Stratagene (La Jolla, CA) for construction of a ZAP Express® Custom cDNA library. The cDNA was inserted into the *Eco* RI/*Xho* I cloning site in the amino-terminal region of the *lacZ* gene in pBK-CMV, the ZAP Express vector (Stratagene).

Plating and titering the λZAP wheat cDNA expression library. The *Escherichia coli* strain, XL1-Blue-MRF′ (Stratagene) was grown on tetracycline selective plates and a single colony was used to inoculate Luria broth (LB) medium (per litre: 10 g tryptone, 5 g yeast extract, 10 g NaCl) supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$. The culture was grown overnight at 37°C with shaking. The culture was spun down at 1000 x *g* for 10 min and the media was discarded. The bacterial pellet was resuspended in 10 mM $MgSO_4$ to an optical density (OD_{600}) of 0.5 at 600 nm. The phage library was serially diluted (1:10,000 to 1:1,000,000) in SM buffer (per litre: 5.8 g NaCl, 2 g $MgSO_4 \cdot 7H_2O$, 50 ml 1 M Tris-HCl (pH 7.5), 5 ml 2% w/v gelatin). One µl of phage was added to 200 µl XL1-Blue-MRF′ cells and incubated for 15 min at 37°C. 3.75 µl of 2 M

Figure 5. Construction and screening of the wheat cDNA expression library. Total RNA was isolated from hard red spring wheat, AC Barrie caryopses harvested at 10-20 days after pollination. Reverse transcription was performed to create cDNA, which was ligated into the *Eco* RI/*Xho* I cloning site in the amino-terminal region of the *lacZ* gene in pBK-CMV, the ZAP Express vector (Stratagene). XL1-Blue-MRF′ *E. coli* was infected with the bacteriophage library (20,000 pfu/150 mm plate). Protein expression was induced by the addition of IPTG. Plaque lifts were made by overlaying the agar with nitrocellulose membranes. The membranes were probed with serum IgG antibodies from diabetic BB rats (pooled, $n = 7$). The secondary antibody was goat anti-rat IgG, Fcγ fragment-specific antibody. Positive clones were detected as dark purple plaques on a background of light pink negative plaques. Screening was repeated until positive phage reached clonality. Single clone excision was performed to allow *in vivo* excision and recircularization of the bacteriophage DNA to form a phagemid. The cDNA inserts were sequenced. Nucleotide and translated BLAST searches of the GenBank and TIGR Wheat Gene Index databases were performed for each sequence.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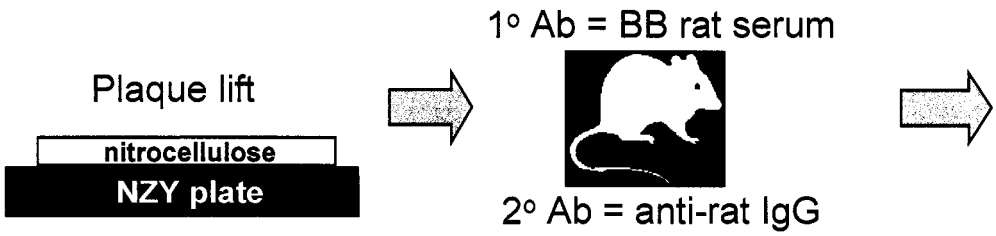
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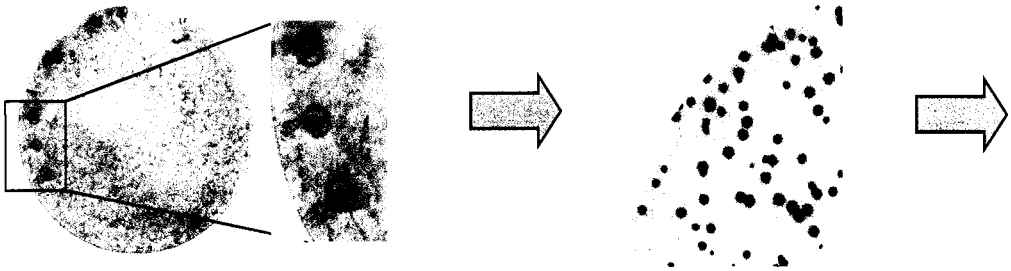
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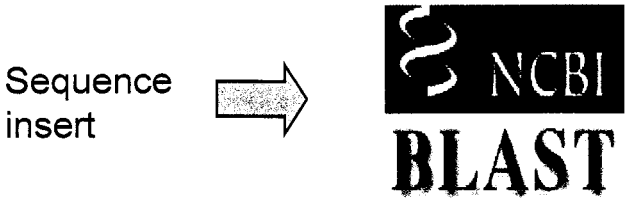

Sequence
insert
NCBI
BLAST

isopropyl β-thiogalactopyranoside (IPTG) was added to the cells and incubated for 15 min at 37°C. NZ amine A (NZY) top agar (per litre: 5 g NaCl, 2 g $MgSO_4 \cdot 7H_2O$, 5 g yeast extract, 10 g casein hydrolyzate, 0.7% w/v agarose, pH 7.5) was heated until boiling and 2 ml aliquots were placed in 15 ml polypropylene tubes and incubated at 48°C. Fifty µl of 250 mg/ml (in dimethylformamide) 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) were added to each tube of melted NZY top agar. The phage and bacteria mix was added to the top agar and immediately poured onto 90 mm x 15 mm NZY agar plates (per litre: 5 g NaCl, 2 g $MgSO_4 \cdot 7H_2O$, 5 g yeast extract, 10 g casein hydrolyzate, 15 g agar) and allowed to solidify. The plates were inverted and incubated overnight at 37°C. The recombinant plaques were white and the nonrecombinant plaques were blue.

Preabsoprtion of diabetic BB rat serum with E. coli lysate. The primary antibody used for screening the library consisted of pooled end-point sera from diabetic BB rats (n = 7; mean age at diabetes onset = 101 ± 18 days; 3 female and 4 male) fed the WP diet from weaning. The BB rat antibodies were preabsorbed with *E. coli* phage lysate to remove *E. coli* specific antibodies. To prepare the *E. coli* lysate, a 100 ml overnight culture of XL1-Blue-MRF′ in LB broth was made. The cells were centrifuged at 1000 x g for 10 min at 4°C. The pellet was resuspended in 3 ml of 50 mM Tris-HCl (pH 8.0) and 10 mM ethylene diamine tetraacetic acid (EDTA). The suspension was freeze-thawed 5 times and sonicated at full power for 6 periods of 20 seconds each at 0°C (Fisher Model 100 Sonic Dismembrator). The extract was centrifuged at 1000 x g for 10 min at 4°C. The supernatant was transferred to a sterile tube and stored at –20°C. For preabsorption of the sera, the *E. coli* lysate was added to serum diluted 1:10 in blocking buffer to a

final concentration of 20% (v/v) and incubated overnight at 4°C. The serum was then diluted further to 1:200 with blocking buffer containing 0.09% sodium azide and stored at 4°C.

Primary screening of the λZAP wheat cDNA expression library. The wheat ZAP Express® Custom cDNA library was plated at a plaque density of 2×10^4 plaque forming units (pfu) per 150 × 15 mm NZY agar plate. 600 µl of XL1-Blue-MRF' *E. coli* (OD_{600} = 0.5) was infected with the appropriate volume of the phage library to achieve the desired pfu density. The mixture was incubated for 15 min at 37°C. Protein expression was induced by the addition of 15 µl of 2 M IPTG followed by a 15 min incubation at 37°C. The mixture was added to 8 ml of prewarmed 48°C NZY top agar and immediately poured onto 150 x 15 mm NZY agar plates. Plates were inverted and incubated overnight at 37°C.

The following day, the plates were overlaid with 0.45 µm, 137 mm, NitroPure nitrocellulose membranes (Osmonics Inc., Minnetonka, MN) and incubated at 37°C for 3 h. An 18 gauge needle was dipped in india ink and used to mark the membrane and agar asymmetrically for later mapping of positive clones. The membranes were removed from the agar plates and washed 4 x 5 min each in Tris-buffered saline (TBS; 100 mM Tris-Cl, pH 7.5, 150 mM NaCl) containing 0.1% Tween20 (TBST; Sigma), with shaking at room temperature. To block nonspecific antibody binding, the membranes were blocked overnight in blocking buffer (5% w/v skim milk powder in TBS). Individual membranes were transferred to 20 ml of the preabsorbed primary antibody, pooled serum from 7 WP-fed diabetic rats diluted 1:200 in blocking buffer, and incubated with shaking for 1 h at 4°C. The primary antibody was collected and the membranes were washed 4 x 5 min

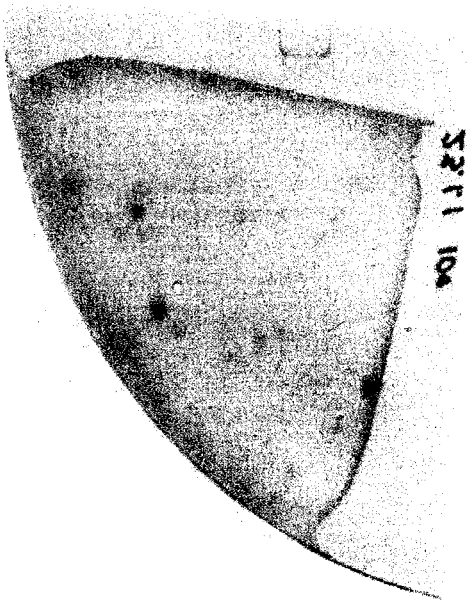
each in TBST and transferred to the secondary antibody, alkaline phosphatase (AP)-conjugated AffiniPure goat anti-rat IgG, Fcγ fragment-specific antibody (Jackson ImmunoResearch, West Grove, PA) diluted 1:5000 in blocking buffer. Rat IgG antibody binding was detected using AP development solution (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) containing nitro-blue tetrazolium chloride (0.3 mg/ml, NBT) and 5-bromo-4-chloro-3-indolyl phosphate (0.15 mg/ml, BCIP). Positive clones were detected as dark purple plaques on a background of light pink negative plaques. Using the India ink marks, the membrane and agar were aligned and positive plaques were located. The positive plaques were cored from the agar using a 1 ml pipette tip. The agar plugs were placed in 500 µl of SM buffer containing 20 µl chloroform and stored at 4°C. The phage diffused from the plug into the buffer.

Secondary and tertiary screening of the λZAP cDNA expression library. All positive clones from the primary screening were screened again using the same sera as above. 1:1 and 1:10 dilutions of the phage were made and 1 µl of the diluted phage was incubated with 200 µl XL1-Blue-MRF' *E. coli* (OD_{600} = 0.5) for 15 min at 37°C. 3.75 µl of 2 M IPTG was added to the mixture and incubated for 15 min at 37°C. The mixture was added to 2 ml of melted NZY top agar and poured immediately onto one quarter of an NZY agar plate. The plates were incubated overnight at 37°C. Nitrocellulose membranes were laid on the plates and were processed in the same manner as for primary screening. Positive clones appeared dark purple on a background of negative light pink plaques (Figure 6). Positive plaques were cored from the agar and stored in SM buffer with chloroform. Screening was repeated until the positive phage had reached clonality.

Figure 6. Identification of positive clones using serum from WP-fed BBdp rats. To determine whether clones identified during the primary screening of the library were positive, secondary and tertiary screening of the clones were performed. Positive clones appeared as dark purple plaques against a background of light pink negative plaques. *Panel A* and *B*, secondary screening of clone WP5.2 and tertiary screening of clone WP23.1.1, respectively. Positive clones were repeatedly screened until they reached clonality.

(A)

(B)



Single clone excision. Single clone excision was performed to allow *in vivo* excision and recircularization of the cloned insert from positive phage, according to the manufacturer's instructions (Stratagene). 200 µl of XL1-Blue-MRF′ (OD_{600} = 1.0) was combined with 250 µl phage stock (>1 x 10^5 phage particles) and 1 µl of the ExAssist helper phage (>1 x 10^6 pfu/µl, Stratagene) and incubated for 15 min at 37°C. Three ml of NZY broth was added and the mixture was incubated overnight at 37°C with shaking. The tube was heated to 68°C for 20 min and spun at 10,000 x *g* for 15 min. The supernatant containing the pBK-CMV phagemid stock was decanted into a sterile container and stored at 4°C. 100 µl or 10 µl of the excised phagemid stock was added to 200 µl XLOLR cells (OD_{600} = 1.0, Stratagene). The mixture was incubated at 37°C for 15 min after which 300 µl of NZY broth was added. The tube was incubated for 45 min at 37°C. 200 µl of the mixture was plated on kanamycin selective LB agar plates and incubated overnight at 37°C. Resistance to kanamycin indicated the presence of the pBK-CMV phagemid.

Phagemid DNA preparation and sequencing. The double-stranded phagemid DNA was isolated using a Plasmid Midi Kit (Qiagen, Mississauga, ON) following the manufacturer's instructions. The cDNA inserts were sequenced at the University of Ottawa Biotechnology Research Institute on a 373 Stretch sequencer (Applied Biosystems, Foster City, CA) using standard T3 forward and T7 reverse primers. For clone WP5212, internal primers were designed to sequence the full length of the cDNA insert (forward primer, 5'-ACCACGGGTTCGTCAAGG-3'; reverse complement primer, 5'-AACACCTCCTGCACCTCC-3').

Immunoscreening wheat clones with individual serum samples

All serum samples were preabsorbed with *E. coli* lysate to eliminate *E. coli* specific antibodies, as described in the aforementioned protocol. Positive phage clones isolated from the wheat cDNA expression library screened with pooled diabetic BB rat serum, clones WP5212, WP12111, WP23112 and WPCON, were screened with individual serum samples.

BB rats. Sera from individual diabetic (n = 7), asymptomatic (no clinical symptoms of diabetes by end of study at day 150; n = 10) BBdp and BBc (n = 9) rats were diluted 1:200 in blocking buffer containing 0.09% sodium azide and used to screen the wheat clones. The secondary antibody was AP-conjugated AffiniPure goat anti-rat IgG, Fcγ fragment-specific antibody (Jackson ImmunoResearch) diluted 1:5000 in blocking buffer.

NOD mice. Prospective sera from individual 80 d female prediabetic (n = 15), asymptomatic (no clinical symptoms of diabetes by 270 d; n = 10) NOD, and 70 d (necropsy) female NOR (n = 7), NOD.Itgb7$^{-/-}$ (n = 12) and NOD.Itgb7$^{-/-}$/L-Sel$^{-/-}$ (n = 8) mice were diluted 1:200 in blocking buffer containing 0.09% sodium azide. The secondary antibody, AP-conjugated goat anti-mouse polyvalent immunoglobulins (IgG, IgA, IgM, (mostly IgG); Sigma), was diluted 1:5000 in blocking buffer.

Human subjects. For the initial case study, the primary antibody was serum from the highly wheat sensitive T1D/CD patient or healthy controls (n = 8) serially diluted 1:400, 1:800, 1:1600 in blocking buffer containing 0.09% sodium azide. The secondary antibody, goat anti-human IgG (Fc-specific) antibody conjugated to AP (Sigma), was diluted 1:20,000 in blocking buffer.

For all samples, the wheat clones were screened in the same manner as for the secondary screening of the library. Antibody binding was detected using AP development solution containing NBT (0.3 mg/ml) and BCIP (0.15 mg/ml). Densitometric analysis of regions of interest on plaque lifts of wheat clones was performed using the Kodak Digital Science™ image station 440CF (Kodak Canada Inc., Toronto ON). Background optical density was measured and subtracted from the value for regions of interest for each plaque lift. The mean intensity/pixel for each region of interest was tabulated. The Kodak image analysis software was standardized using a Kodak control scale T-14 (Kodak Canada Inc.). The density scale has 14 steps ranging from 0.04 to 2.05 with each step being 0.15 optically denser than the adjacent step. The linear range for intensity per pixel values was between density 0.04 and 0.8 (Pearson product-moment correlation $r = 0.91, p = 0.013$; data not shown). The observed intensity per pixel values fell within this range.

Assessment of pancreatic islet damage

All histological analyses were performed on coded samples. Bouin's fixed pancreatic sections were prepared by Peter Smyth in the lab of Dr. Olga Pulida (Pathology Section, Toxicology Research Division, Food Directorate, Health Canada, Ottawa, Canada). The hematoxylin and eosin (H&E)-stained sections of pancreas were evaluated at ×100 magnification and confirmed at ×200 magnification using an Axiolab microscope (Zeiss, Mississauga, ON). Subjective overall rating of pancreatic islet inflammation (insulitis) was performed using the following scale: 0, normal islet appearance; 1, infiltration at islet periphery only; 2, infiltration concentrated in islet periphery with infiltration in the islet core; 3, infiltration concentrated in one-third of the

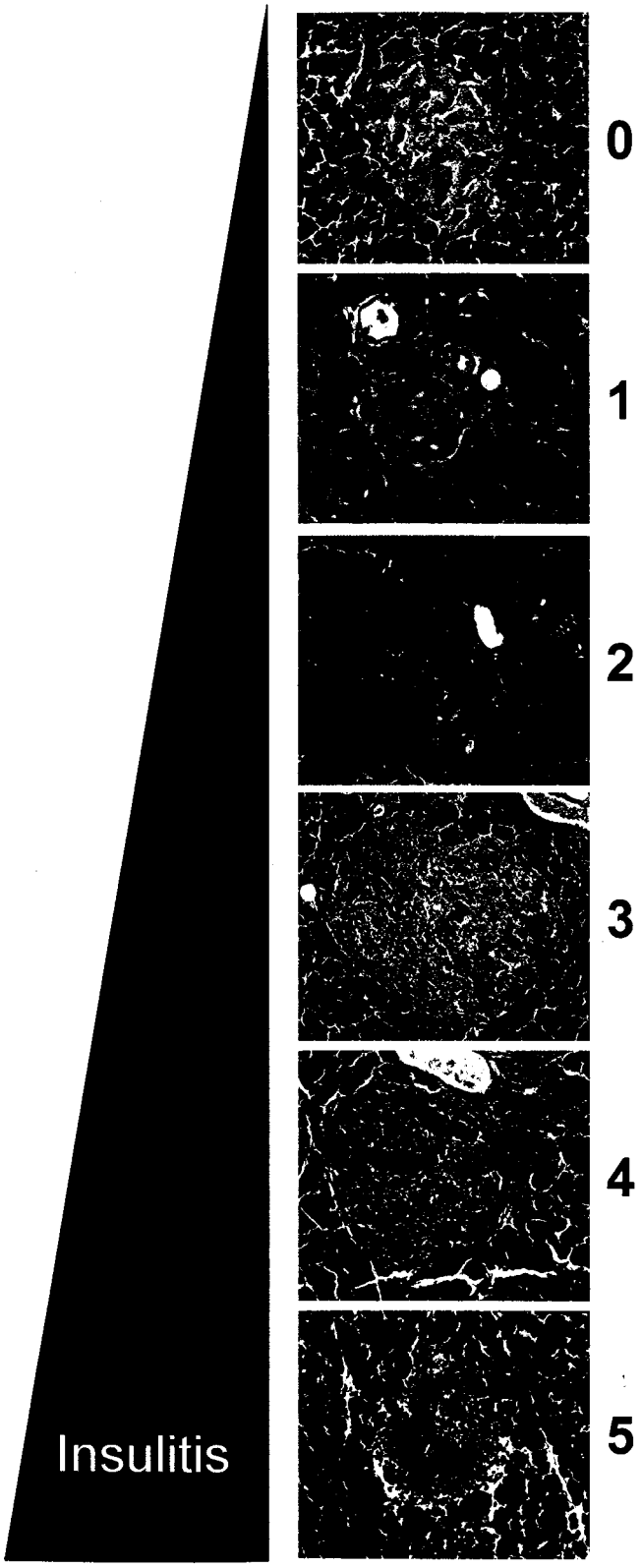
islet core; 4, infiltration concentrated in up to one-half of the islet core; 5, end stage islets with widespread β-cell destruction and/or core filled with infiltrating mononuclear cells (Figure 7) (162). The mean of 10 islets per animal was used for an overall insulitis score. The degree of target tissue inflammation was measured as the percent of infiltrated islets per section for each animal.

Expression of recombinant WP5212 in Sf21 insect cells

IPLB-Sf21 insect cell line. Insect *Spodoptera frugiperda* (Sf21) cells were grown at 27 °C in complete Grace's insect cell culture medium (supplemented; Gibco, Burlington, ON) containing 10% fetal bovine serum (Sigma), penicillin (50 units/ml; Sigma), and streptomycin (50 μg/ml; Sigma).

Recombinant WP5212-baculovirus construct. The WP5212-baculovirus was constructed and generously provided by Dr. Markus Walz from the lab of Dr. Hubert Kolb (German Diabetes Research Institute, Heinrich Heine University of Düsseldorf, Germany). Dr. Walz generated the recombinant WP5212-baculovirus using the BacPAK6 baculovirus expression system (BD Biosciences Clontech, Mississauga, ON). WP5212-baculovirus infected cells were lysed in phosphate buffered saline (PBS) and sent to our lab for analysis. One-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1D SDS-PAGE) and Western blots of the lysate were probed with WP5212 antibody positive diabetic rat serum (confirmed by plaque lifts, Study 2) as the primary antibody to confirm WP5212 protein expression.

Figure 7. Insulitis rating. The degree of pancreatic islet inflammation (insulitis) was evaluated using the following subjective rating: 0, normal islet appearance; 1, infiltration in islet periphery only; 2, infiltration concentrated in islet periphery with infiltration in the islet core; 3, infiltration concentrated in one-third of the islet core; 4, infiltration concentrated in up to one-half of the islet core; 5, end stage islets with widespread β cell destruction and/or core filled with infiltrating mononuclear cells (162). The mean score of 10 islets per animal was used for an overall insulitis score. Inflammation of the islets was also measured as the percent of infiltrated islets per pancreatic section (not shown).


Insulitis
0
1
2
3
4
5

Isolation of clonal recombinant viruses. In order to isolate clonal recombinant WP5212-baculovirus, 1 x 10^6 Sf21 insect cells (70-80% confluent in 35 mm plates) were infected with insect cell lysate (provided by Dr. Markus Walz) serially diluted 1:10, 1:100 and 1:1000 in complete Grace's medium. As a positive control, 1 x 10^6 insect cells were infected with BacPAK6 virus diluted 1:100,000 or 1:1,000,000 (according to the manufacturer's manual; Clontech). Uninfected cells acted as a negative control. The cells were left for 1 h at room temperature to allow for infection of the cells. The medium was aspirated and 1.5 ml of 37°C prewarmed 1% SeaPlaque agarose (2% agarose mixed 1:1 with complete medium) was added to each well. Once the agarose overlay was set, 1.5 ml of complete Grace's medium was added to each well. The dishes were incubated at 27°C for 5 days. One ml of 0.03% neutral red was added to each well and the dishes were incubated for 2 h at 27°C. The stain was aspirated and the dishes were inverted in the dark overnight. A total of 7 viral plaques were observed in the wells infected with insect cell lysate. The plaques were picked and added to 0.5 ml of complete Grace's medium. The selected plaques were vortexed and left overnight at 4°C to allow the viruses to diffuse into the medium.

Preparation of passage one virus stock. 35 mm plates were seeded with 5 x 10^5 cells in complete Grace's medium. 100 µl of each plaque pick was used to infect cells as above. BacPAK6 (parental) and AcMNPV C6 (wild type) virus stocks were used as positive controls at a dilution of 1:10,000. Uninfected cells acted as a negative control. The cells were incubated at 27°C for 4 days. The supernatant containing the passage one virus stock was centrifuged at 1,000 x *g* for 5 min to remove cells and debris. The supernatant was transferred to a new tube and the passage one virus stocks were stored at

4°C, until confirmation of WP5212 recombinant viral clones by 1D SDS-PAGE and Western blotting, at which point they were titered, aliquotted and maintained at -70°C.

Evaluation of recombinant WP5212-baculovirus clones. The cells from above were scraped into PBS and pelleted at 1,000 x *g* for 1 min. The pellet was washed once in PBS and centrifuged again. The pellet was resuspended in 100 µl of PBS and 100 µl 2X SDS-PAGE loading buffer (100 mM Tris-Cl, pH 6.8, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, 200 mM β-mercaptoethanol). Samples were maintained at -20°C. Clones expressing the WP5212 were confirmed by 1D SDS-PAGE and Western blotting (see method below). Recombinant WP5212-baculovirus clones were titered following the plaque assay procedure described in the method for isolation of clonal recombinant viruses (above). Clone 4 was determined to express WP5212 and was used for all further analyses (see Figure 24, *panel A* and *B*). The titer of the Passage One clone 4 virus stock was determined to be 3.5×10^7 pfu/ml.

Amplification and preparation of Passage Two virus stock of recombinant WP5212 viruses. Fifty ml suspension insect cell cultures in log phase growth at a cell density of 5×10^5 cells/ml were infected with passage one virus stock at a Multiplicity of Infection = 0.1 (MOI = 0.01, defined as 0.1 pfu for every cell in the suspension culture). The suspension cultures were incubated at 27°C for 5 days. The cells were centrifuged at 1,000 x *g* for 5 min. The supernatant, the Passage Two virus stock, was aliquotted and stored at -70°C. The passage two virus stock was titered following the plaque assay protocol described in the method for isolation of clonal recombinant viruses (above). The titer of the Passage Two clone 4 virus stock was determined to be 9.3×10^7 pfu/ml.

Expression of recombinant WP5212 in insect cells. 12.0 x 10^6 log phase insect cells (from a spinner cutlure that had been inoculated with 2 x 10^5 cells/ml and that had reached a density between 0.7 and 1 x 10^6 cells/ml) were plated in a 150 cm^2 flask and incubated at 27°C for 2 h. The cells were infected with WP5212-baculovirus or parental BacPAK6 at an MOI = 10. Uninfected cells acted as a negative control. The cells were incubated for 2 days at which point cells were harvested and centrifuged at 1,000 x *g* for 5 min. The pellet was washed twice and resuspended in PBS. The cells were lysed by sonication. Samples were stored at -20°C.

WP5212 polyclonal serum production

Peptide synthesis. Antigenic WP5212 peptides were predicted by Sigma-Genosys technical services (proprietary software). Two WP5212 specific peptides were synthesized for the purpose of polyclonal WP5212 antisera production (Sigma-Genosys, The Woodlands, TX). Peptide 1, a 77% pure 16 residue peptide (CRDTFNLLEQRPKIAN), was conjugated to the carrier protein Keyhole-limpet hemocyanin for immunization. Peptide 2 was a 51% pure 15 residue peptide (RGDEAVEAFLRMATA). The purity was determined by mass spectral and HPLC analyses performed by Sigma-Genosys.

Polyclonal antisera production. The antibody production was performed by Sigma-Genosys. Pre-immune serum was collected from two rabbits, after which they were each co-immunized with 200 μg of peptides 1 and 2 in Complete Freund's Adjuvant (day 0). The rabbits were co-immunized with 100 μg of peptides 1 and 2 in Incomplete Freund's Adjuvant on days 14, 28, 42, 56 and 70. Production bleeds from both rabbits

were collected on day 40, 63 and 77. WP5212-specific polyclonal antibody production was assessed by 1D SDS-PAGE and Western blotting of WP5212-baculovirus infected insect cell lysate. To allow for the comparison of production bleeds to pre-immune serum, blots were mounted onto a Hoefer decaprobe (Amersham Pharmacia Biotech, San Francisco, CA) for multi-antibody screening.

1D SDS-PAGE and Western blotting

Preparation of 10% SDS-PAGE gels. 1D SDS-PAGE and Western blotting were performed using Hoefer mini VE electrophoresis and electrotransfer units (Amersham Pharmacia Biotech). To make one 10% separation gel, 2.5 ml of 40% acrylamide/bis solution, 37.5:1 (2.6% C) (Bio-Rad, Hercules, CA), 2.5 ml of 4X Tris-Cl/SDS, pH 8.8 (1.5 M Tris-Cl, 0.4% SDS) and 5 ml H_2O were mixed and degassed under vacuum for 5 min. 50 µl of 10% ammonium persulfate (APS) and 10 µl of N,N,N′,N′-tetramethylethylenediamine (TEMED) were added, swirled and poured immediately. To make the 6% stacking gel for one gel, 0.5 ml of 40% acrylamide/bis solution, 37.5:1 (2.6% C) (Bio-Rad), 0.83 ml of 4X Tris-Cl/SDS, pH 6.8 (0.5 M Tris-Cl, 0.4% SDS) and 2 ml H_2O were mixed and degassed under vacuum for 5 min. 16.7 µl of 10% APS and 3.3 µl of TEMED were added, swirled and poured immediately.

Protein sample preparation. Protein samples from WP5212-baculovirus infected insect cell lysate were quantified using the Bio-Rad DC Protein Assay, following the manufacturer's protocol. This is a colorimetric assay that is similar to the Lowry protein concentration assay (163), but which has been modified to allow for the presence of detergent in the protein sample (Bio-Rad technical manual). Samples were diluted in 2X

SDS sample loading buffer and denatured by heating for 5 min at 100°C. Ten μg of total protein was added to each well. A prestained molecular mass marker was used (BenchMark Prestained Protein Ladder, Invitrogen, Burlington, ON).

Protein electrophoresis, electrotransfer and immunoblotting. Protein samples were resolved by electrophoresis on 10% SDS-PAGE gels at 200 V in electrophoresis running buffer (25 mM Tris-base, 192 mM glycine, 0.01% SDS, 0.1 mM phenylmethylsulfonyl fluoride (PMSF, Sigma)). The proteins were electrotransferred from the gel to pure nitrocellulose membranes (pore size 0.45 μm, Bio-Rad) at a constant current of 300 mA (approximately 25 V) for 1 h in transfer buffer (25 mM Tris-base, 192 mM glycine, 20% methanol, pH 8.3). The membrane was stained with Ponceau S red (Sigma) to ensure protein transfer to the nitrocellulose membrane. The membrane was washed in TBST for 4 x 5 min. Non-specific antibody binding sites were blocked with 5% (w/v) skim milk blocking buffer overnight at 4°C. The membranes were placed in primary antibody diluted in blocking buffer for 1 h with shaking. The membranes were washed 4 x 5 min each in TBST and placed in secondary antibody diluted in blocking buffer for 1 h. The membranes were washed 2 x 5 min with TBST and 2 x 5 min with TBS. Antibody binding was detected using alkaline phosphatase development solution containing NBT (0.3 mg/ml) and BCIP (0.15 mg/ml). Densitometric analysis of bands of interest was performed using the Kodak Digital Science™ image station 440CF (Kodak Canada Inc.). For each blot, background optical density was measured and subtracted from the value for the band of interest. The mean intensity/pixel for each band of interest was tabulated.

Antibodies used for screening immunoblots. To determine the identity of the over-expressed protein in the WP5212-baculovirus infected insect cell lysate, WP5212 antibody positive diabetic BB rat serum was diluted 1:200 in blocking buffer containing 0.09% sodium azide and used to confirm WP5212 protein expression in insect cell lysate samples sent from Dr. Markus Walz (Düsseldorf, Germany). The corresponding secondary antibody was AP-conjugated AffiniPure goat anti-rat IgG, Fcγ fragment-specific antibody (Jackson ImmunoResearch) diluted 1:5000 in blocking buffer. WP5212-baculovirus clones were also screened with serum from the highly wheat sensitive patient with diabetes and celiac disease diluted 1:2000 in blocking buffer containing 0.09% sodium azide. The secondary antibody, goat anti-human IgG (Fc-specific) antibody conjugated to AP (Sigma), was diluted 1:20,000 in blocking buffer.

For confirmation of WP5212 expression in baculovirus infected insect cells, pre- and post-immunization polyclonal rabbit serum samples were diluted 1:500 or 1:1000 in blocking buffer containing 0.09% sodium azide and used as the primary antibody. The secondary antibody, goat anti-rabbit immunoglobulins conjugated to alkaline phosphatase (Dako Diagnostics Canada Inc., Mississauga, ON) was diluted 1:1000 in blocking buffer.

To determine the level of WP5212 antibodies in individual patients and study subjects, serum was diluted 1:200 in blocking buffer containing 0.09% sodium azide and used as the primary antibody. The secondary antibody, goat anti-human IgG (Fc-specific) antibody conjugated to AP (Sigma), was diluted 1:20,000 in blocking buffer. After alkaline phosphatase development, the blots were stained with Ponceau S red to confirm the location of WP5212 on the blot allowing for the positive identification of WP5212 antibody negative and positive patients for image analysis purposes.

Preabsorption of WP5212 antibodies with desmin and vimentin. Serum from the highly wheat sensitive patient with both type 1 diabetes and celiac disease was diluted 1:2000 in blocking buffer. Five or 50 μg of bovine desmin (from bovine lenses, Sigma) or recombinant human vimentin (Progen, Heidelberg, Germany) were added to the diluted patient serum and incubated overnight at 4°C with end-over-end rotation. Unabsorbed serum and serum preabsorbed with 50 μg of β-lactoglobulin B (from bovine milk, Sigma) were used as negative controls. The preabsorbed serum was used as the primary antibody for probing 1D Western blots of WP5212-baculovirus infected insect cell lysate (10 μg/lane).

Construction and immunoscreening of a WP5212 B cell epitope library

Construction of the WP5212 epitope library. The Novatope Library Construction System (Novagen, Madison, WI) for characterizing B cell epitopes within antigenic polypeptides was used to map sites of antibody binding within WP5212. DNase I digestion of the 6X His-WP5212/pET17b expression construct (see Appendix I) containing the complete ORF of WP5212 was performed using the DNase Shotgun Cleavage kit (Novagen) following the manufacturer's instructions. The DNase I digestion reaction was set up by adding 10 μg of WP5212/pET17b plasmid DNA to DNase I (0.0015, 0.001, 0.0007 or 0.0005 U/μl), DNase I buffer (0.05 M Tris-HCl pH7.5, 0.05 mg/ml BSA), 10 mM $MnCl_2$ in a total volume of 10 μl. The reactions were incubated at room temperature for 10 min, after which 2 μl of 6X Stop Buffer (100 mM EDTA, 30% glycerol and tracking dye) was added. Samples were analyzed by agarose gel electrophoresis on a 2% gel. The samples containing DNA fragments ranging in size

from 30-150 bp were pooled and run on a 2% agarose gel. The band corresponding to 30-150 bp fragments was excised from the gel and equilibrated by adding 1X volume of the gel to 10X agarase buffer (100 mM Bis Tris-HCl pH 6.5, 10 mM Na_2EDTA; New England Biolabs, Beverly, MA) to make 1% gel. The agarose was melted at 65°C for 10 min. The sample was cooled to 42°C and the molten agarose was incubated with 2 U of β-agarase I (per 200 µl 1% agarose, New England Biolabs) at 42°C for 1 h. The DNA solution was extracted sequentially with one volume Tris-EDTA (TE)-buffered phenol (Sigma), one volume of 1:24:1 phenol:chloroform:isoamyl alcohol (phenol:CIAA; Sigma) and 1 volume CIAA (Sigma). The DNA was precipitated by adding 0.1 volume of 3 M NaOAc and 2 volumes ethanol. The sample was placed at -70°C for 1 h. It was centrifuged at 12,000 x *g* for 15 min, washed once with 1 volume of 70% ethanol, centrifuged at 12,000 x *g* for 5 min and the pellet was dried. The DNA was resuspended in 30 µl TE buffer. The DNA concentration was determined at A_{260}.

The resulting oligonucleotides (ranging 30–150 bp in length) were blunt-ended and tailed with a single dATP using the Single dA Tailing Kit (Novagen). The DNA ends were blunted by combining 1 µg of the DNA with flushing buffer (0.05 M Tris-HCl pH 8.0, 5 mM $MgCl_2$, 0.1 mg/ml BSA), 0.1 mM dCTP, dGTP and dTTP, 1 mM dATP, 5 mM DTT and 1 U T4 DNA polymerase in a total volume of 25 µl. The reaction mixture was shaken gently and incubated at 11°C for 20 min. The reaction was stopped by heating to 75°C for 10 min. A single 3′ dA residue was added to the blunt ends by adding 10X dA tailing buffer (100 mM Tris-HCl pH 9.0, 0.5 M KCl, 0.1% gelatin, 1% Triton X-100) and 1.25 U of *Tth* DNA polymerase to the entire flushing reaction and bringing the reaction volume to 85 µl. A positive dA tailing control was set up by adding 100 ng of a

positive control 36mer to flushing buffer (0.05 M Tris-HCl pH 8.0, 5 mM $MgCl_2$, 0.1 mg/ml BSA), 0.1 mM dCTP, dGTP and dTTP, 1 mM dATP, 10X dA tailing buffer and 0.75 U *Tth* polymerase in a total reaction volume of 42.55 µl. The reactions were incubated at 70°C for 15 min. One volume of CIAA was added, the sample was vortexed for 60 s and centrifuged at 12,000 x *g* for 1 min. The aqueous phase was added to a fresh tube.

The oligonucleotides were ligated into the pSCREEN T-vector. The ligation reaction was made by adding 6 ng sample DNA to 5 mM DTT, 0.5 mM ATP, 50 ng pSCREEN T-vector, 2 U T4 DNA ligase in a total reaction volume of 10 µl. The sample was mixed gently and incubated overnight at 16°C. The DNA was transformed into NovaBlue (DE3) *E. coli* competent cells following the protocol provided (Novagen). In brief, 1 µl of the ligation reaction was added to 20 µl of cells and stirred gently. The samples were placed on ice for 30 min. The samples were heated for 30 s at 42°C and then placed on ice for 2 min. Cells were grown for 1 h at 37°C with shaking. 50 µl of each transformation was spread on antibiotic selective LB agar plates containing 50 µg/ml ampicillin and 15 µg/ml tetracycline. The plates were inverted and incubated overnight at 37°C.

Screening the WP5212 epitope library with serum from a highly wheat sensitive T1D/CD patient. The library of random, overlapping inserts expressed by transformed cells was screened by colony immunoscreening with WP5212 antibody positive serum. The epitope library was plated at a density of approximately 1000 colony forming units (cfu) on LB plates containing 50 µg/µl ampicillin and grown overnight at 37°C. The plates were overlaid with pure nitrocellulose membranes (Osmonics). The orientation of

the filter on the plate was marked by an 18 gauge needle dipped in India ink. After one min of contact, the membranes were carefully lifted off and placed in a chloroform vapor chamber for cell lysis. The chamber was sealed with Saran wrap and left for 15 min at room temperature. The membranes were removed from the chamber and placed colony side up on a piece of Whatman paper saturated with colony denaturing solution (20 mM Tris-HCl pH 7.9, 6 M urea, 0.5 M NaCl) for 15 min at room temperature. The membranes were placed in 5% (w/v) nonfat skim milk blocking buffer and incubated with shaking for 30 min. The membranes were washed twice for 15 min each with TBST and placed in the primary antibody diluted in blocking buffer for 30 min. Colonies were immunoscreened for protein expression using WP5212 positive serum from the highly wheat sensitive case study patient with both type 1 diabetes and celiac disease diluted 1:2,000 in blocking buffer. The membranes were washed 3 x 10 min each with TBST and placed in secondary antibody, anti-human IgG Fc-specific conjugated to alkaline phosphatase (1:20,000) for 30 min. The membranes were washed 3 x 10 min each with TBST and placed in AP developer containing 30 mg/ml NBT and 15 mg/ml BCIP. Dark purple colonies were deemed positive in contrast to negative pink colonies.

Identification of epitopes. Bacterial colonies expressing immunoreactive peptides were selected and plasmid inserts were sequenced by using primers complementary to flanking regions of the cloning site in pSCREEN T-vector. Positive colonies were picked and grown overnight at 37°C with shaking. Plasmid DNA was prepared using the Wizard Plus SV miniprep kit following the manufacturers instructions (Promega). Sequencing of the inserts was performed by the Ottawa Genome Centre DNA Sequencing Facility (Ottawa, ON). Insert sequences were aligned with the WP5212 ORF using Align Plus 5,

version 5.01 software from the Clone Manager Professional Suite (Scientific and Educational Software, Durham, NC).

DNA and amino acid homology searches

Nucleotide and translated Basic Local Alignment Search Tool (BLAST) searches of the GenBank (164) and The Institute for Genomic Research (TIGR) Wheat Gene Index (TIGR Gene Index Databases, The Institute for Genomic Research, Rockville, MD 20850, http://www.tigr.org/tdb/tgi, accessed April 15, 2004) databases were performed (165). Amino acid homologies were retrieved with and without the low complexity filters using the Blosum 62 homology matrix. When searching for homologies for sequences less than 35 amino acids in length sequence homology matrix PAM-30, which is recommended when looking for short, nearly-exact matches, was used (165).

Three-dimensional modeling of WP5212

The translated amino acid sequence of WP5212 was submitted to SWISS-MODEL (http://www.expasy.org/swissmod/SWISS-MODEL.html) for three-dimensional rendering (166-169). This program finds similarities of the target sequence with sequences of known three-dimensional structures. Templates are selected based on sequence identity greater than 25% and projected model size larger than 20 residues. It also detects domains that can be modeled based on unrelated templates. The program generates ProModII input files for the target sequence and generates models, which are subjected to algorithms for energy minimization. SWISS-MODEL uses a protein database derived from the Protein Data Bank (PDB), termed ExPDB, which is used by

BLAST to search for suitable modeling templates. The three-dimensional structure of WP5212 was visualized using Deep View-SwissPDB Viewer (http://www.expasy.org/spdbv/) (169).

HLA peptide binding predictions

The full length amino acid sequence of WP5212 was submitted to SYFPEITHI (http://syfpeithi.bmi-heidelberg.com/scripts/MHCServer.dll/home.htm), a MHC class I and II peptide binding motif search program (170). T cell epitope predictions were made by the evaluation of 15mer peptides across the entire WP5212 protein sequence for the diabetes-associated HLA class II molecule haplotypes, HLA-DRB1*0301 and HLA-DRB1*0401.

Statistics

Survival analysis was performed using the Kaplan-Meier Product-Limit Estimator. The effect of different diets on survival (diabetes incidence) was analyzed using the log-rank test. Means were calculated and differences among sample populations were compared using one-way analysis of variance (ANOVA) and Least Significant Difference (LSD) or Scheffe's post hoc tests (LSD<Scheffe's for statistical stringency). Fisher's exact test (two-tailed) was used to compare frequencies. Pearson product-moment correlation was used to determine r and p values. All statistics were performed using STATISTICA Version 4.5 (StatSoft Inc., 1993, Tulsa OK).

RESULTS

Study 1: Immunoscreening a wheat cDNA expression library to identify diabetes-related antigenic wheat proteins in the BBdp rat

It has been shown that the incidence of diabetes is dramatically increased in BBdp rats and NOD mice fed cereal based diets relative to animals fed a protective hydrolyzed casein diet (see Figure 4 and 16). These results suggest that disease outcome for a subset of susceptible individuals is dependent on the protein component of the diet. Susceptible individuals could have an abnormal immune response to particular WG antigens, which in turn could initiate diabetes or enhance the disease process. A humoral immune response could ensue causing the development of antibodies to the culpable dietary proteins. These antibodies can be used to identify antigenic, diabetes-related wheat proteins using immunoscreening methods.

Characterization of the wheat cDNA expression library. The unamplified cDNA expression library consists of approximately 1.0 x 10^6 recombinant phages (personal communication, Dr. Mike Kobrin, Stratagene). The titer of the amplified library is approximately 2 x 10^8 pfu/ml, representing the 10^6 recombinants of the original library (Table 1). The library is approximately 2.8% nonrecombinant (Table 1) and contains cDNA inserts with a mean length of 1.4 kb (personal communication, Dr. Mike Kobrin, Stratagene; data not shown).

Three antigenic wheat clones isolated from a wheat cDNA expression library. BBdp rats were weaned onto the semipurified wheat protein diet at 23 days of age and monitored for diabetes development. The primary screening of the library, using pooled

Table 1. Characterization of the amplified wheat cDNA expression library.

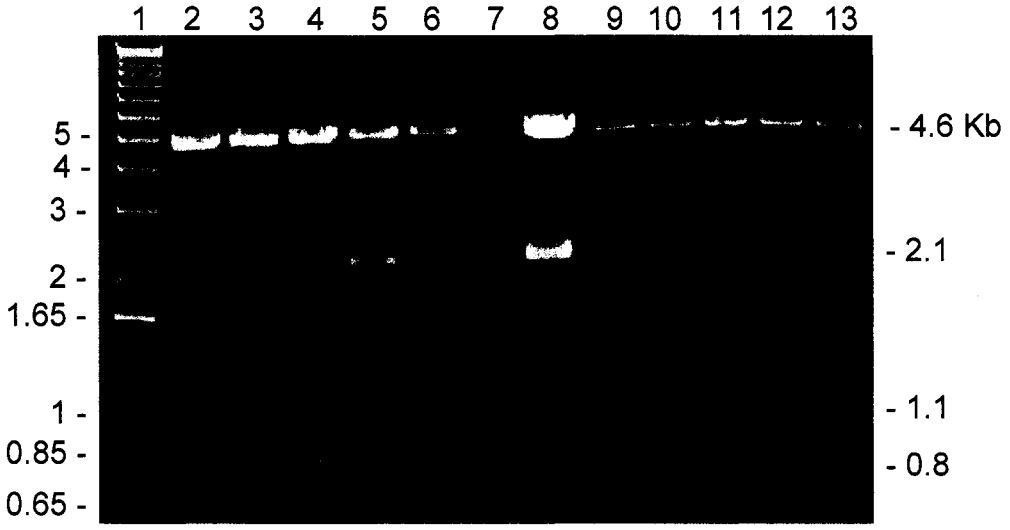
Trial	Titer (x 10^8 pfu/ml)	% non-recombinant
1	1.76	4.5
2	2.08	0.47
3	2.13	3.4
mean ± 1 SD	1.99 ± 0.2	2.79 ± 2.08

diabetic BB rat serum ($n = 7$; 3 female and 4 male; age at disease onset ranged between 73 and 121 d), yielded 48 positive clones. In the secondary screening, eight clones were found to be positive when compared to non-specific antibody binding and were repeatedly screened until they reached clonality (Figure 6, *panel A* and *B*). The eight clones could be categorized into three groups based on cDNA insert size (2.1, 1.1, and 0.8 kb; Figure 8, *panel A*). Sequencing the cDNA inserts confirmed the presence of three distinct sets of positive clones (Table 2). Representative clones, WP5212, WP12111 and WP23112, from each distinct set were used for all further analyses (Figure 8, *panel B*).

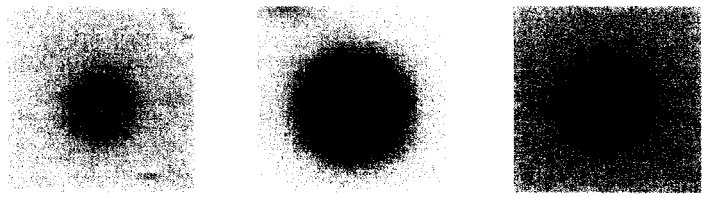
Nucleotide and translated BLAST searches of the GenBank and TIGR Wheat Gene Index databases were performed (Table 2). Each clone was linked by a 13 bp linker-primer 3' to 96 bp of the N-terminal portion of the β-galactosidase gene. Clone WP5212 contained a 1890 bp open reading frame (ORF), including the fusion gene and linker. Within the β-galactosidase ORF was the in-frame complete WP5212 cDNA sequence. The WP5212 cDNA ORF was 1767 bp in length. It shared 100% homology across the entire open reading frame of the *Triticum aestivum* (wheat) EST clone TC103916 with the tentative annotation Homologue to globulin Beg1 precursor (Table 2; Figure 9, *panel A* and *B*). It shared 90% identity across 1387 nucleotides with the *T. aestivum* wheat storage protein (Glb1) gene (accession number M81719.1; Table 2). The expected translated amino acid sequence was 629 amino acids in length, including the β-galactosidase fusion protein, and 588 amino acids alone. The WP5212 open reading frame shared 80% identity across 642 amino acids with the *T. aestivum* wheat storage protein (accession number AAA34269.1; Table 2; Figure 10), Glb1 and the barley

Figure 8. Restriction digest analysis of clones isolated from the wheat cDNA expression library immunoscreened with antibodies from WP-fed diabetic BB rats. *Panel A*, three distinct sets of clones approximately 0.8 (lanes 2 to 4), 2.1 (lanes 4 to 8) and 1.1 kb (lanes 9 to 13) in length were isolated from the wheat cDNA expression library. The 4.6 kb band is the pBK-CMV vector. *Panel B*, plaque lifts of antigenic wheat proteins, WP23112, WP5212 and WP12111. Upon sequencing the cDNA inserts, all clones within the respective sets were shown to be identical. Therefore, one clone from each set was used for all further analyses, WP23112 (0.8 kb), WP5212 (2.1 kb) and WP12111 (1.1 kb).

(A)



(B)



WP23112 WP5212 WP12111

Table 2. Identification and characterization of clones isolated from a wheat cDNA expression library using serum from diabetic BB rats.

Clone	DNA homology, % identity/no. bp[a]	Amino acid homology, % identity/no. amino acids[a]	ORF length[b] (bp)	Putative protein length (amino acids)[c]
WP5212	*Triticum aestivum* (wheat) EST TC103916 Homologue to globulin Beg1 precursor, 100%/1767 *T. aestivum* (wheat) storage protein (Glb 1) gene, 90%/1387	 *T. aestivum* (wheat) storage protein, 80%/642	1890 1890	 629
WP12111	*T. aestivum* EST TC179215 weakly similar to unknown protein {Arabidopsis thaliana}, partial (26%), 90%/673	Unknown protein, *A. thaliana*, 63%/144	789	262
WP23112	*T. aestivum* EST TC176537 similar to GP|9293891|dbj|BAB01794.1||AB026654 contains similarity to uridylate kinase gene {A. thaliana}, partial (5%), 95%/651	Putative protein, *A. thaliana*, 63%/144	624	207
WPCON	Ascorbate peroxidase (*Hordeum vulgare*) 91%/326	Ascorbate peroxidase (*H. vulgare*), 96%/86	366	121

[a] BLASTn and BLASTx sequence homology searches were performed using the GenBank and TIGR Wheat Gene Index data bases.
[b] The open reading frame contains 95 bp from the 5' β-galactosidase gene and 13 bp from the cDNA linker.
[c] Determined using Clone Manager 6® (Sci-Ed Software (2002) website address: www.scied.com/ses_cm6.htm, Scientific and Educational Software, Durham, NC). Includes the β-galactosidase N-terminal fusion protein.

Figure 9. DNA sequence homology between WP5212 and the EST wheat clone TC103916. *Panel A*, clone WP5212 shares 100% nucleotide sequence homology across the open reading frame with the EST clone TC103916 tentatively annotated Homologue to Beg1 precursor (TIGR Wheat Gene Index). Homologous nucleotides in the EST TC103916 are represented as dots. *Panel B*, regions of homologous nucleotide sequence are represented as blue bars. Open reading frames are indicated by black arrows. Alignments were made using the Clone Manager Professional Suite software (Scientific and Educational Software, Durham, NC).

(A)

```
WP5212      1    gatagttcaaccatggcgaccagaggcagagcaaccatccctctcctcttcctcctgggcacaagccttctcttcgccgcggctgtttcggcctcccatg
TC103916    34   ............................................................................................................

WP5212      101  acgaggaggaggacaggcgcggtgggcgctcgcttcagcggtgcgtgcagcggtgccagcaggaccggccgcggtactctcatgcccggtgcgtgcagga
TC103916    134  ............................................................................................................

WP5212      201  gtgccgggacgaccagcagcagcacggaaggcacgagcaggaggagcagggccgcgggcatggccggcacggcgaggggggagcgtgaggaggagcagggc
TC103916    234  ............................................................................................................

WP5212      301  cgtggccgtgggcggcgcggccagggagagcgtgaggaggagcagggccgtggacgtgggcggcgccggcgagggagagcgtgatgaggagcacggggatg
TC103916    334  ............................................................................................................

WP5212      401  gccggcggccgtacgtgttcggcccgcgcagcttccgccgcatcatccggagcgaccacgggttcgtcaaggcccttcgcccgttcgacgaagtgtccag
TC103916    434  ............................................................................................................

WP5212      501  gctcctccgggggcatcaggaactaccgtgtcgccatcatggaggtgaacccgcgcgcgttcgtcgtgccgggactcacggacgcagacggcgtcggctac
TC103916    534  ............................................................................................................

WP5212      601  gtcgctcaaggcgaggggggtgctgacggtgatcgagaacggcgagaagcggtcctacaccgtcaggcaaggcgatgtgatcgtggcgccggcggggtcca
TC103916    634  ............................................................................................................

WP5212      701  tcatgcacctggccaacaccgacggccggaggaagctggtcatcgccaagattctccacaccatctccgtccccggcaagttccagtatttctcggccaa
TC103916    734  ............................................................................................................

WP5212      801  gcctctcctcgctagtttgagcaaacgcgtgctcacagcggcgttaaagacctcggatgagcggctgggtagtctcttgggcagccgccaaggcaaggag
TC103916    834  ............................................................................................................

WP5212      901  gaggaggagaagtccatctccatcgtccgcgcgtcagaggagcagctccgcgagctgcgtcgccaggcgtccgagggtgaccagggccaccactggcctc
TC103916    934  ............................................................................................................

WP5212      1001 tccccccgttccgcggcgactcgcgcgacaccttcaacctcctggagcagcgccccaagatcgccaaccgccatggccgcctctacgaggccgacgcccg
TC103916    1034 ............................................................................................................

WP5212      1101 tagcttccacgcccctcgcccaacacgacgtccgcgtcgccgtggccaacatcacgccgggttctatgaccgcgccctacctgaacacccagtcgttcaag
TC103916    1134 ............................................................................................................

WP5212      1201 ctcgccgtcgtgctggaaggcgagggcgaggtggagatcgtctgcccgcacctcggccgcgacagcgagcgccgcgagcaagagcacggcaagggcaggt
TC103916    1234 ............................................................................................................

WP5212      1301 ggaggagcgaggaagaggaggacgaccggcggcagcaacgccgacgcgggtccggctccgagtcggaggaggagcaggaccagcagaggtacgagacggt
TC103916    1334 ............................................................................................................

WP5212      1401 ccgcgcgcgggtgtcgcgcggctcggcgttcgtggtgccccccggccacccggtggtggagatcgcctcgtcccgcggcagcagcaacctccaggtggtg
TC103916    1434 ............................................................................................................

WP5212      1501 tgcttcgagatcaacgccgagaggaacgagcgggtgtggctcgccgggaggaacaacgtgatcgccaagctggacgaccccgcccaggagctcgccttcg
TC103916    1534 ............................................................................................................

WP5212      1601 gcaggcccgcgagggaggtgcaggaggtgttccgcgccaaggatcagcaggacgagggcttcgtcgccggacccgagcagcagcaggagcatgagcgcgg
TC103916    1634 ............................................................................................................

WP5212      1701 ggaccgccgccgtggtgaccgcgggcgcggcgacgaagccgtggaggcgttcctgaggatggcaaccgccgcgctctgaggcggcaaggccgctgttgtt
TC103916    1734 ............................................................................................................

WP5212      1801 aagtgaatgtgtgagctggagcccgtgccatttgagagctgaacttgtatgtgtgtgtaagtttgtcagtacgcgggagtagcataaaataagtcgtggca
TC103916    1834 ............................................................................................................

WP5212      1901 cgggctcagtacgatgatgtaagttgcgtacctaccttctaccaaggcatgcatgcccaacataaataaacacaagggcgttgcgcctctttttcagtaa
TC103916    1934 ............................................................................................................

WP5212      2001 aaaaaaaaa
TC103916    2034 .........
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(B)

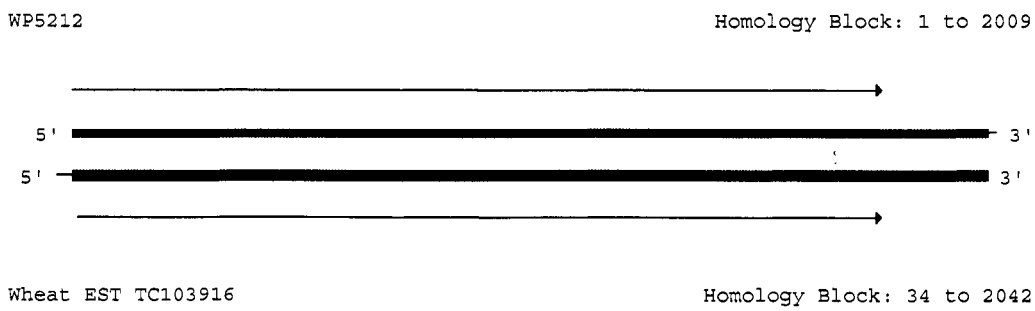
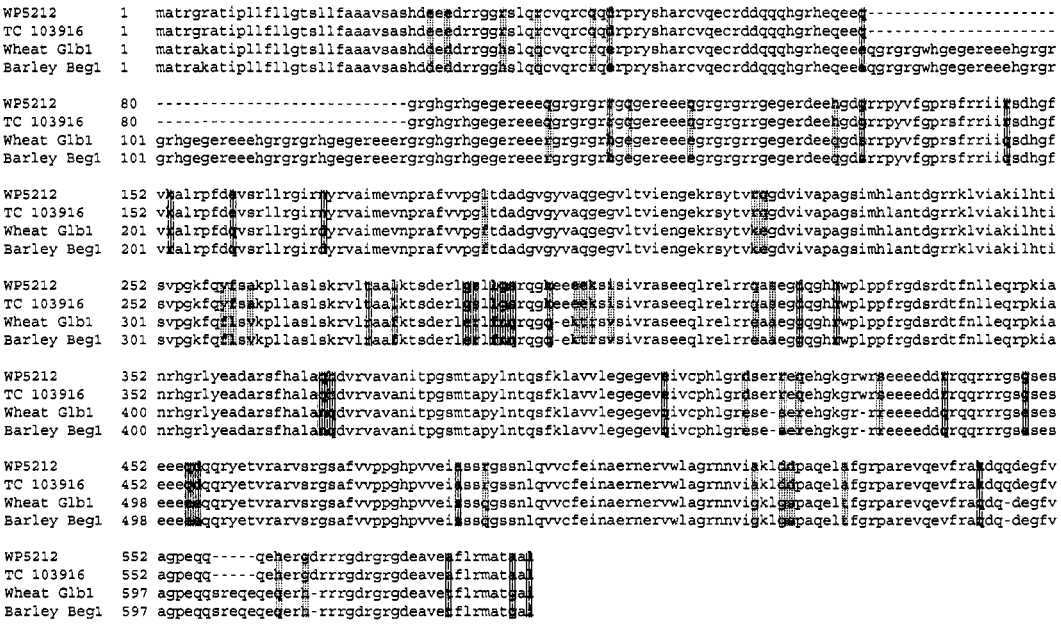
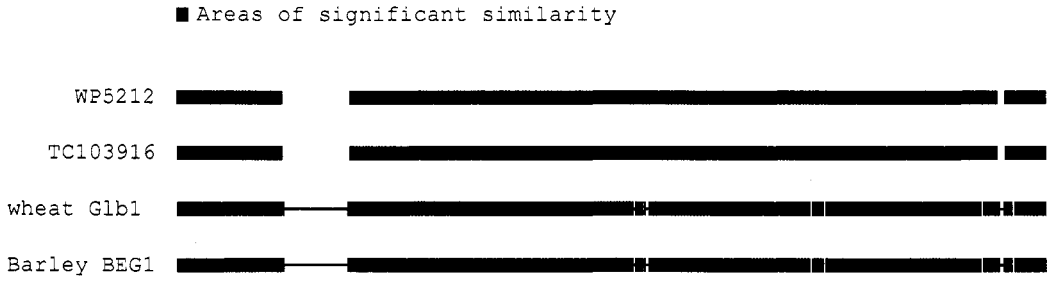


Figure 10. Amino acid homology among WP5212, EST TC103916, the wheat storage globulin, Glb1, and the barley embryo globulin, Beg1. *Panel A*, amino acid alignments of the predicted amino acid sequences of WP5212 and the wheat EST clone TC103916, as well as the wheat storage globulin, Glb1 and the barley embryo globulin, Beg1, were performed using the Clone Manager Professional Suite software. WP5212 and TC103916 share 100% amino acid homology, and 80% homology with both Glb1 and Beg1. Gaps in the protein sequences are represented by dashes. Differences in amino acid sequence are shaded in pink. *Panel B*, a pictorial representation of the amino acid alignment between the predicted amino acid sequences of clone WP5212 and EST TC103916, and the sequences of Glb1 and Beg1 proteins is shown. Regions of homology are represented by blue bars; regions of amino acid differences are represented by thin black lines; gaps in amino acid sequence are represented by spaces.

(A)


WP5212
TC 103916
Wheat Glb1
Barley Beg1

(B)


Areas of significant similarity
WP5212
TC103916
wheat Glb1
Barley BEG1

embryo globulin, Beg1 (accession number AAA32936.1; Figure 10). Glb1 and Beg1 are 100% homologous for both DNA and amino acid sequences.

The ORF for cDNA clone WP12111 was 789 bp and coded for a putative gene product, including the β-galactosidase fusion, of 262 amino acids, of which 225 originate from wheat cDNA (Table 2). The nucleotide sequence shared 90% identity across 673 nucleotides with the wheat EST clone TC179215 and 63% identity across 144 amino acids with an unknown *Arabidopsis thaliana* protein (accession number AAK25945.1).

Clone WP23112 had an ORF of 624 bp coding for a putative product of 207 amino acids (Table 2). WP23112 shared 95% identity across 651 nucleotides with the wheat EST clone TC176537 and 63% identity across 144 amino acids with a putative *A. thaliana* protein (accession number CAB75463.1). Clone WP23112 also shared 100% identity across 511 nucleotides with a clone from a Brevor mature wheat embryo ABA library (accession number BE515816).

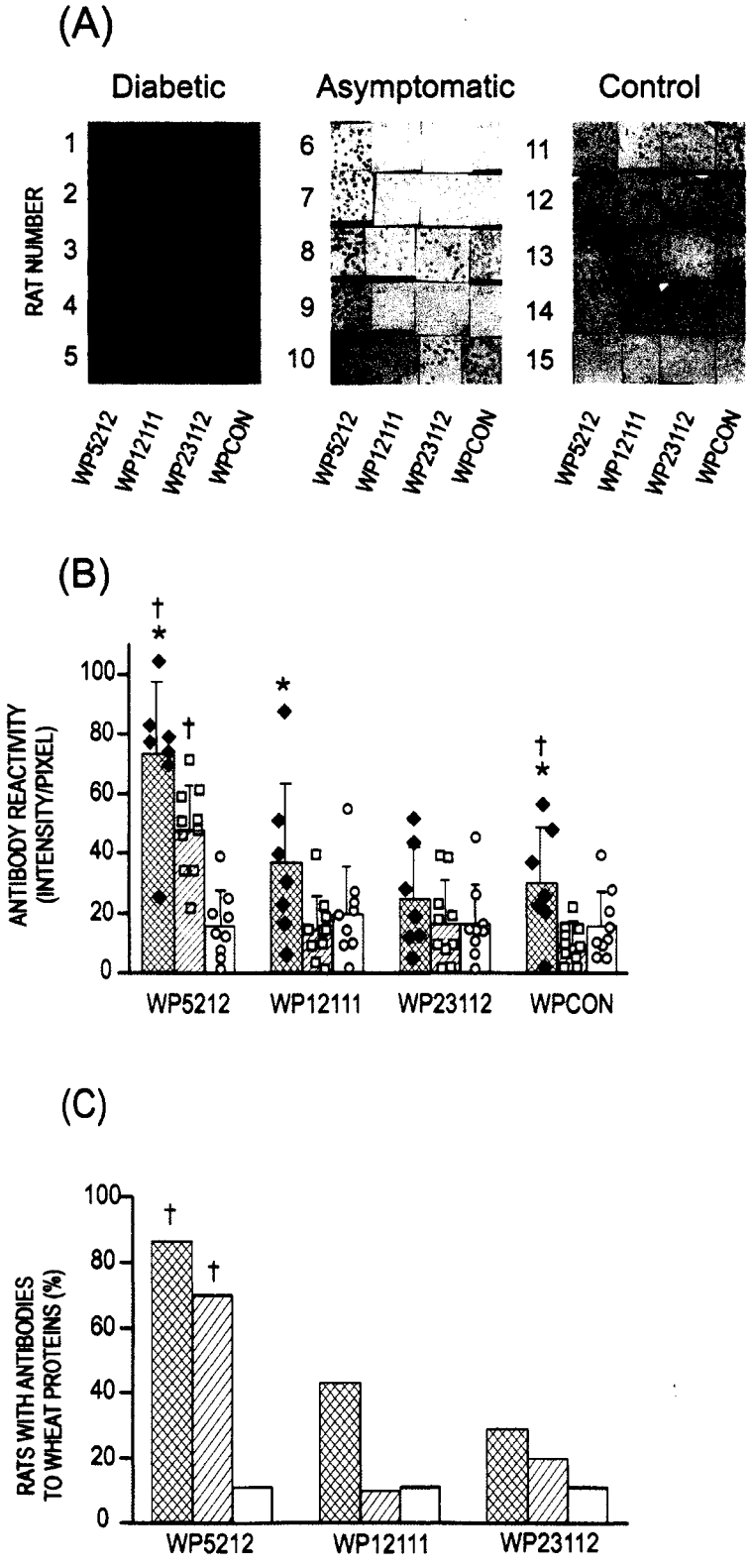
A clone was randomly chosen from the library to represent background antibody binding. This clone, termed WPCON, had a 366 bp long ORF and an expected expression product size of 121 amino acids (Table 2). WPCON shared 91% identity across 326 nucleotides with barley ascorbate peroxidase mRNA (*Hordeum vulgare*, accession number AF411228.1) and shared 96% identity across 86 amino acids with the *H. vulgare* ascorbate peroxidase protein (accession number AAL08496.1).

Study 2: Antibody reactivity to candidate type 1 diabetes-related wheat proteins and their correlation to islet damage in the BBdp rat

We propose that the identified wheat proteins are diabetes-related. Therefore, the production of antibodies to these dietary proteins will be restricted to BBdp rats and can be used as markers of disease development and/or progression. To test this hypothesis, it was necessary to determine the extent to which diabetic animals develop antibodies to the wheat proteins and whether antibody production was associated with disease outcome.

Diabetic rats have increased frequency and intensity of antibody reactivity to clone WP5212. To determine whether antibody reactivity to WP5212, WP12111, and WP23112 was related to diabetes risk, the clones were screened with serum antibodies from individual diabetic ($n = 7$; mean age at diabetes onset = 101 ± 18 days; 3 females, 4 males), asymptomatic ($n = 10$; no clinical symptoms of diabetes by end of study; mean age at end of study = 149 ± 1 days; 4 females, 6 males) BBdp, and BBc ($n = 9$; mean age at end of study = 128 ± 1 days; 4 females and 5 males) rats (Figure 11, *panels A* and *B*; (49)). Antibody reactivity was measured by densitometry and is reported as intensity/pixel. Antibody reactivity to WP5212 in diabetic rats (73.1 ± 24.4 mean intensity/pixel) was significantly higher than in asymptomatic (47.5 ± 14.6 mean intensity/pixel; ANOVA/LSD, $p = 0.005$) and control (15.5 ± 11.5 mean intensity/pixel; $p = 10^{-6}$) rats (Figure 11, *panel B*). Asymptomatic BBdp rats also had increased antibody reactivity to WP5212 compared with control rats ($p = 0.0004$). Diabetic rats had higher antibody reactivity to WP12111 (36.7 ± 26.9 mean intensity/pixel) than asymptomatic rats (14.3 ± 11.1 mean intensity/pixel; $p = 0.02$). Antibody reactivity to WP23112 did not differ among the rat groups. Diabetic rats had increased antibody reactivity to WPCON

Figure 11. Antibody reactivity to clones isolated from the wheat cDNA expression library screened with serum from diabetic, asymptomatic and control BB rats. *Panel A*, five representative plaque lifts of clones WP5212, WP12111, WP23112, and WPCON screened with serum from individual diabetic, asymptomatic, or control rats. *Panel B*, mean antibody reactivity (mean intensity/pixel) ± SD to the recombinant wheat proteins in diabetic ($n = 7$, cross-hatched bars), asymptomatic ($n = 10$, hatched bars), or control ($n = 9$, open bars) BB rats is shown. Individual values for diabetic (diamonds), asymptomatic (squares), or control (circles) rats are shown. ANOVA/LSD; † indicates significant difference vs. control rats, $p \leq 0.0004$; * indicates significant vs. asymptomatic rats, $p \leq 0.005$. *Panel C*, the frequency of diabetic (cross-hatched bars), asymptomatic (hatched bars), and control (open bars) BB rats with positive antibody reactivity to the wheat proteins is shown. A positive antibody level was defined as an antibody reactivity greater than the mean antibody reactivity plus 2 SD (38.5 mean intensity/pixel) of WPCON screened with control rat serum. Fisher's exact (two-tailed) test; † indicates significant difference vs. control rats, $p \leq 0.02$. Modified and reprinted with permission from *Journal of Biochemistry* vol. 278, pp. 54-63 (2003).


(A)
Diabetic
Asymptomatic
Control
RAT NUMBER
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
WP5212
WP12111
WP23112
WPCON
(B)
ANTIBODY REACTIVITY (INTENSITY/PIXEL)
100
80
60
40
20
0
WP5212
WP12111
WP23112
WPCON
(C)
RATS WITH ANTIBODIES TO WHEAT PROTEINS (%)
100
80
60
40
20
0
WP5212
WP12111
WP23112

(29.8 ± 18.4 mean intensity/pixel) compared with asymptomatic (9.4 ± 7.2 mean intensity/pixel, $p = 0.002$) and control (15.6 ± 11.4 mean intensity/pixel; $p = 0.03$) rats. Antibody reactivity to WP12111, WP23112 or WPCON was not different between asymptomatic BB rats and BBc rats. Antibody reactivity in serum from BBc rats was not different among any of the proteins analyzed, suggesting that this level represented nonspecific (background) antibody reactivity.

The frequency of rats with antibodies to wheat proteins was determined (Figure 11, *panel C*). A positive antibody level was defined as an antibody reactivity value greater than the mean intensity plus 2 SD for WPCON screened with control serum (38.5 intensity/pixel units). More diabetic (85.7%, $p = 0.009$) and asymptomatic (70%, $p = 0.02$) rats had antibodies to WP5212 than control rats (11.1%), but there was no difference in frequency of antibody reactivity to WP5212 between diabetic and asymptomatic rats. Although diabetic rats (42.9%) tended to be more frequently WP12111 antibody positive than asymptomatic (10%) and control (11.1%) rats, there was no significant difference in frequency of antibody reactivity to either WP12111 or WP23112 among the rat groups.

Antibody reactivity to clone WP5212 correlates with islet inflammation and damage. To determine whether antibody reactivity to the cloned wheat proteins correlated with damage to the target tissue, the proportion of islets infiltrated with mononuclear cells was calculated. Also, the mean insulitis score (overall islet damage) was determined using a subjective scoring system with scores ranging from 0 to 5 (Figure 7). An insulitis score was calculated as the mean insulitis score from 10 islets per pancreatic section. A relationship with diabetogenesis was considered to occur when both

percent infiltration (degree of inflammation) and mean insulitis score (overall islet damage) showed a significant correlation with antibody reactivity to a protein. Diabetic rats had significantly fewer islets (39 ± 19 islets) than both asymptomatic (60 ± 29 islets; $p \leq 0.03$) and control (72 ± 13 islets; $p \leq 0.002$) rats (Table 3). There was no difference in total islet number between asymptomatic and control rats. Diabetic rats had a higher percent of infiltrated islets (63.4% ± 20.6) and mean insulitis score (2.9 ± 1) compared with both asymptomatic (41.4% ± 21.5, $p = 0.02$; 1.3 ± 0.7, $p = 0.0001$) and control (5.0% ± 5.3, $p = 10^{-6}$; 0.2 ± 0.1, $p < 10^{-7}$) rats. In asymptomatic rats, the percent of infiltrated islets was higher, as was the mean insulitis score compared with control rats ($p = 0.0001$ and $p = 0.002$). A positive correlation was observed between antibody reactivity to WP5212 and percent of infiltrated islets (Pearson product-moment correlation $r = 0.81, p = 10^{-6}$; Figure 12, *panel A*) and mean insulitis score ($r = 0.78, p = 3 \times 10^{-6}$; Figure 12, *panel B*). There was no correlation between antibody reactivity to WP12111 or WP23112 and percent of islets infiltrated and mean insulitis score (Figure 12, *panels C* to *F*).

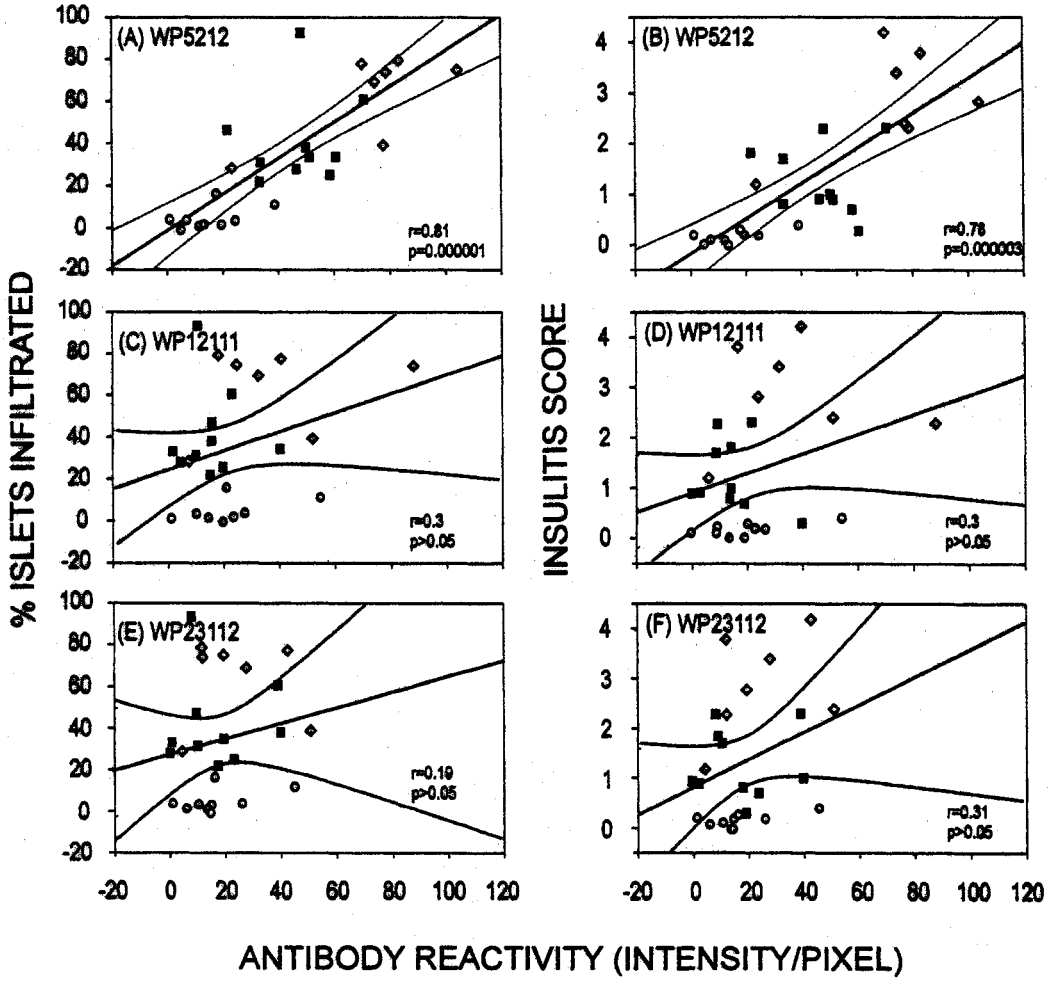
Prediabetic BB rats have increased antibody reactivity to wheat proteins compared with BBc rats. To determine whether diabetes-prone rats develop antibodies to WP5212, WP12111 and WP23112 in the prediabetic period, the proteins were immunoscreened with 70 d prospective serum samples from WG-fed rats. Both prediabetic (n = 10, of which 7 were used in Study 2; 30.8 ± 16.6 mean intensity/pixel) and asymptomatic (n = 12, of which 9 were used in Study 2; 35.3 ± 20.4 mean intensity/pixel) rats developed antibodies to WP5212 with increased reactivity compared with control rats (n = 9, all of which were used in Study 2; 13.5 ± 8.3 mean

Table 3. Number of islets and inflammation in the pancreas of rats whose serum was used to screen wheat clones.

BB rat	Total no. islets	% islets infiltrated	Mean insulitis score
Diabetic (n = 7)	39 ± 19[a,c]	63.4 ± 20.6[b, c]	2.9 ± 1.0[b, c]
Asymptomatic[d] (n = 10)	60 ± 29	41.4 ± 21.5[c]	1.3 ± 0.7[c]
Control (n = 9)	72 ± 13	5.0 ± 5.3	0.2 ± 0.1

[a] All values are mean ± S.D.
[b] Significantly different from asymptomatic (one-way ANOVA/LSD, $p \leq 0.03$).
[c] Significantly different from control (one-way ANOVA/LSD, $p \leq 0.002$).
[d] Asymptomatic refers to animals without clinical symptoms of diabetes by age 150 days.

Figure 12. Antibody reactivity to the WP5212 clone is strongly associated with islet inflammation and damage. The correlation between the percent of islets infiltrated (*left column*) or insulitis score (*right column*) and antibody reactivity (mean intensity/pixel) to three recombinant wheat proteins in diabetic (diamonds), asymptomatic (squares), or control (circles) rats are shown. The Pearson Product-Moment correlation r and p values are indicated. Modified and reprinted with permission from *Journal of Biochemistry* vol. 278, pp. 54-63 (2003).


(A) WP5212
r=0.81
p=0.000001
(B) WP5212
r=0.76
p=0.000003
(C) WP12111
r=0.3
p>0.05
(D) WP12111
r=0.3
p>0.05
(E) WP23112
r=0.19
p>0.05
(F) WP23112
r=0.31
p>0.05
% ISLETS INFILTRATED
INSULITIS SCORE
ANTIBODY REACTIVITY (INTENSITY/PIXEL)

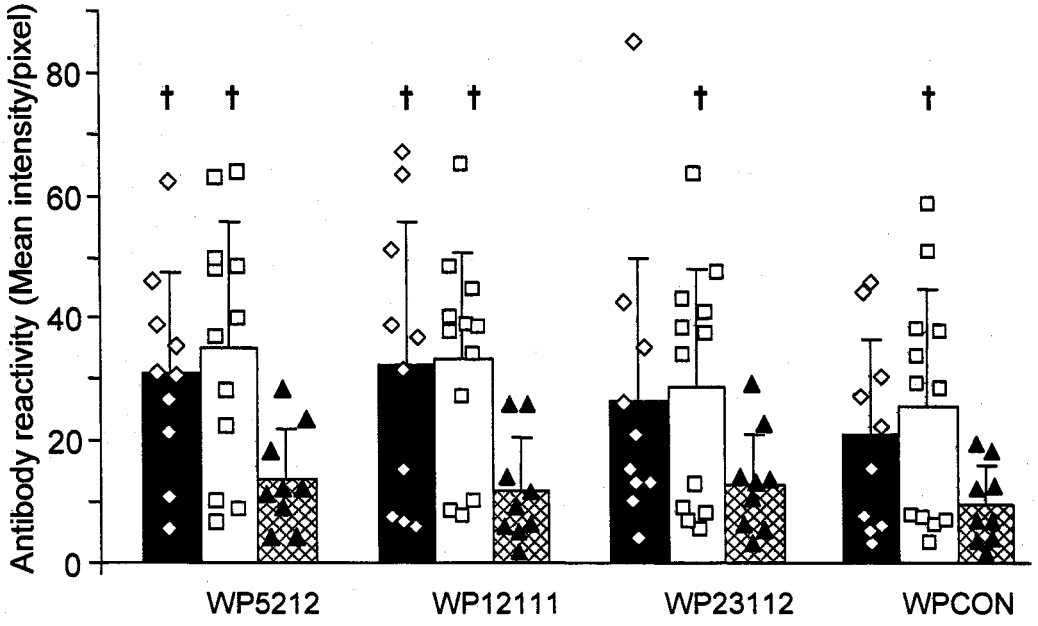
intensity/pixel; $p \leq 0.04$; Figure 13, *panel A*). Also, prediabetic (32.6 ± 23.2 mean intensity/pixel) and asymptomatic (32.4 ± 17.4 mean intensity/pixel) rats had increased WP12111 antibody reactivity compared with control rats (11.9 ± 8.8 mean intensity/pixel; $p \leq 0.04$; Figure 13, *panel A*). There was no significant difference in antibody reactivity to these two proteins between prediabetic and asymptomatic animals. Asymptomatic animals had increased antibody reactivity to WP23112 (28.6 ± 19.5 mean intensity/pixel) and WPCON (25.7 ± 19.0 mean intensity/pixel) compared with control animals (12.7 ± 8.3 and 9.5 ± 6.4 mean intensity/pixel; $p \leq 0.04$) but prediabetic animals did not (26.3 ± 23.5 and 20.8 ± 15.9 mean intensity/pixel; Figure 13, *panel A*).

The frequency of prediabetic rats with antibodies to wheat proteins was determined (Figure 13, *panel B*). A positive antibody level was defined as an antibody reactivity value greater than the mean intensity plus 2 SD for WPCON screened with control serum (22.4 mean intensity/pixel). More prediabetic (80%) and asymptomatic (75%) rats had antibodies to WP12111 than control (20%) rats ($p \leq 0.04$). There was no difference in frequency of antibody reactivity to WP12111 between prediabetic and asymptomatic rats. Prediabetic (70%) and asymptomatic (66.7%) rats tended to be more frequently positive for antibodies to WP5212 compared with control (20%) rats, but this was not significant, likely due to small sample sizes. Similarly, prediabetic (60%) and asymptomatic (58.3%) rats tended to be more frequently positive for antibodies to WP23112 compared with control (20%) rats, but again this was not significant.

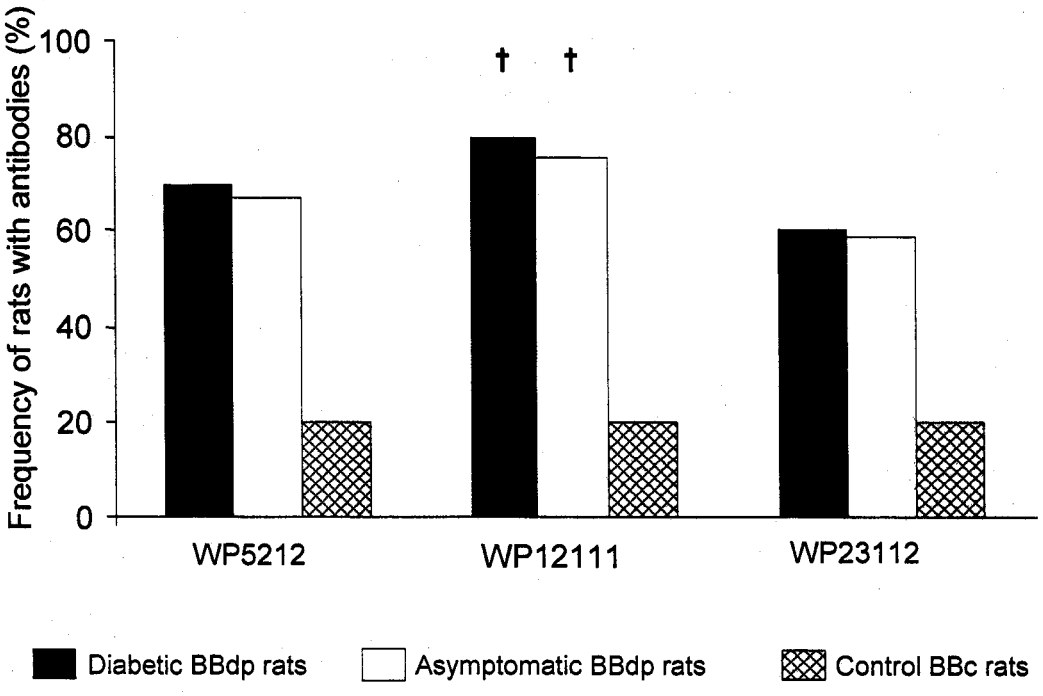
Development of WP5212 antibodies is dependent on a wheat-based diet. To examine whether development of antibodies to WP5212 is specific to the semipurified WG diet, WP5212 was screened with serum from diabetic ($n = 7$) rats fed the protective

Figure 13. 70d prediabetic BB rats have increased antibody reactivity to WP5212 compared with control, but not asymptomatic rats. *Panel A*, mean antibody reactivity (mean intensity/pixel) ± SD to the recombinant wheat proteins screened with 70d prediabetic ($n = 10$, black bars), asymptomatic ($n = 12$, open bars), or control ($n = 9$, cross-hatched bars) BB rats are shown. Individual values for prediabetic (diamonds), asymptomatic (squares), or control (triangles) rats are shown. ANOVA/LSD; † indicates significant difference vs. control rats, $p \leq 0.04$. *Panel B*, the frequency of prediabetic (black bars), asymptomatic (open bars), and control (cross-hatched bars) BB rats with positive antibody reactivity to the wheat proteins is shown. A positive antibody level was defined as antibody reactivity greater than the mean intensity plus 2 SD (22.4 mean intensity/pixel) of WPCON screened with 70d control rat serum. Fisher's exact (two-tailed) test; † indicates significant difference vs. control rats, $p \leq 0.04$.

(A)


Antibody reactivity (Mean intensity/pixel)
0
20
40
60
80
WP5212
WP12111
WP23112
WPCON

(B)


Frequency of rats with antibodies (%)
0
20
40
60
80
100
WP5212
WP12111
WP23112
Diabetic BBdp rats
Asymptomatic BBdp rats
Control BBc rats

HC diet. Diabetic rats fed the WG diet (n = 7, used in Study 2) had significantly higher antibody reactivity than HC-fed diabetic (73.1 ± 24.4 vs. 17.5 ± 10.7 mean intensity/pixel; $p < 10^{-6}$; Figure 14, *panel A*). There was no difference in antibody reactivity to WP5212 between diabetic or asymptomatic HC-fed rats. The frequency of diabetic rats with positive WP5212 antibody reactivity was higher in animals fed the WG diet (85.7 vs. 0% in HC; $p < 10^{-6}$; Figure 14, *panel B*).

WP5212 homologues exist in other cereal and vegetable plants. The correlation between antibody reactivity to WP5212 and damage to the target tissue allowed us to focus our studies on this wheat protein. Other dietary plant proteins, such as from soybean (116)(Rastigar and Scott, unpublished data), have been implicated in the development of diabetes. Therefore, it was of interest to determine whether WP5212 has homologues in other cereal species, as these too could be associated with diabetes. A directed search of TIGR Gene Index databases of specific cereal and vegetable plant species was performed (Table 4). It was found that WP5212 shared significant nucleotide homology with 7S globulin genes, specifically Glb1-like vicilins in barley (77% across 1831 bp), rice (70% across 1542 bp), maize (66% across 1650 bp), sorghum (70% across 1290 bp) and sunflower (54% across 805 bp). WP5212 also shared nucleotide homology with a gene in rye (57% across 620 bp). WP5212 shared nucleotide homology with a putative histone deacetylase from soybean (58% across 346 bp) and a putative vicilin precursor in tomato (56% across 399 bp).

WP5212 shares homology with immunomodulatory plant proteins and self proteins. It is possible that immunomodulatory plant proteins engage the immune system through a mechanism based on shared molecular structure. Therefore, we searched for

Figure 14. Antibody reactivity to WP5212 is dependent on a wheat-based diet in BBdp rats. *Panel A*, mean antibody reactivity (mean intensity/pixel) ± SD to the recombinant wheat proteins screened with WP-fed diabetic ($n = 7$, black bars) or HC-fed diabetic ($n = 7$, open bars) BB rats is shown. Individual values for WP-fed diabetic (diamonds) or HC-fed diabetic (triangles) rats are shown. ANOVA/LSD; † indicates significant difference vs. HC-fed diabetic rats, $p < 10^{-6}$. *Panel B*, the frequency of WP-fed diabetic (black bars) and HC-fed diabetic (white bars) BB rats with positive antibody reactivity to the wheat proteins is shown. A positive antibody level was defined as an antibody reactivity greater than the mean intensity plus 2 SD (38.5 mean intensity/pixel see Figure 11) of WPCON screened with WP-fed control rat serum. Fisher's exact (two-tailed) test; † indicates significant difference vs. HC-fed diabetic rats, $p < 10^{-6}$.

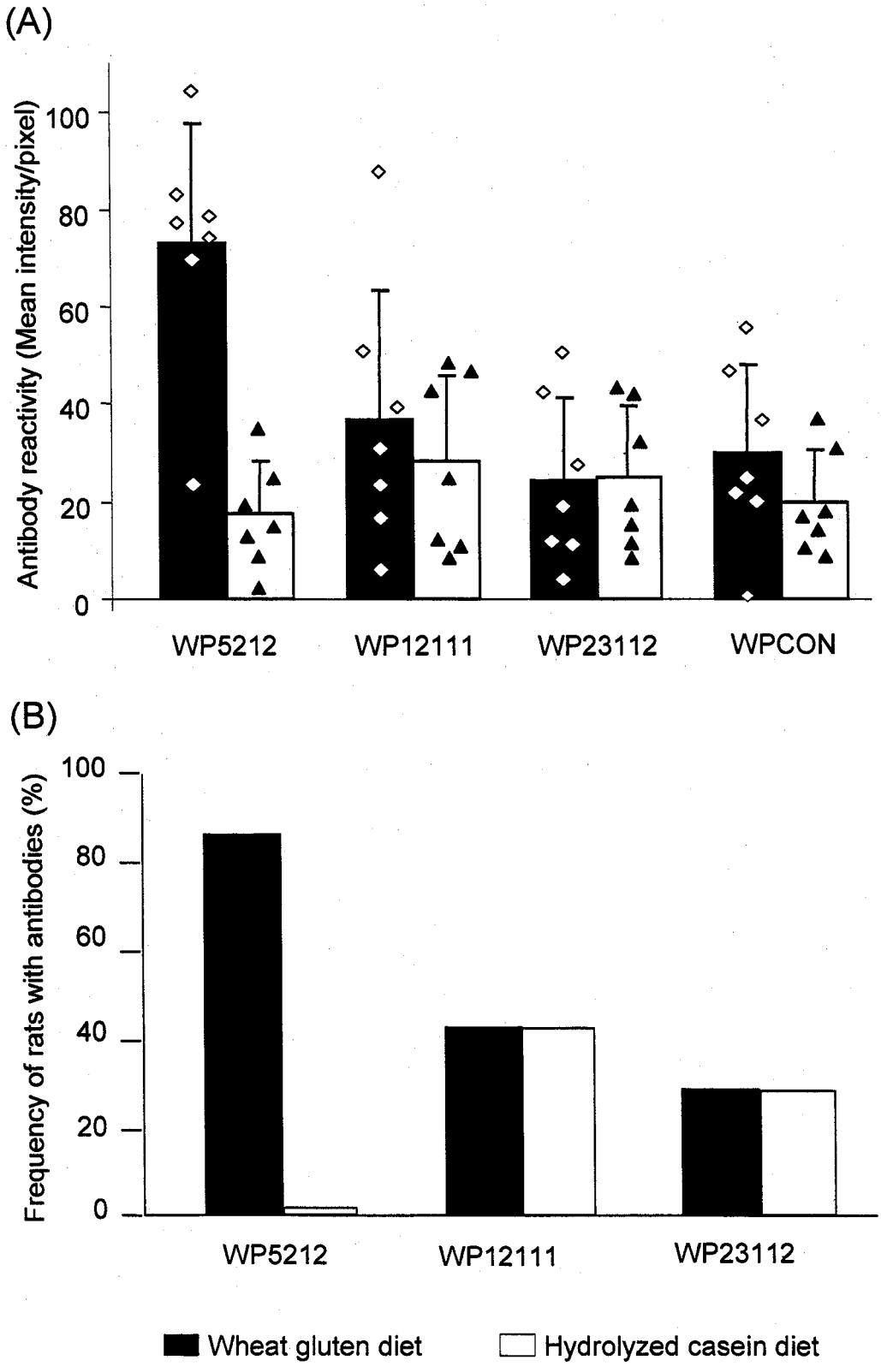

(A)
Antibody reactivity (Mean intensity/pixel)
100
80
60
40
20
0
WP5212
WP12111
WP23112
WPCON
(B)
Frequency of rats with antibodies (%)
100
80
60
40
20
0
WP5212
WP12111
WP23112
Wheat gluten diet
Hydrolyzed casein diet

Table 4. DNA homologues to clone WP5212 in cereal and vegetable plants.

Plant species Common name	EST Clone	% identity/no. bp	Expect value
Triticum aestivum Wheat	Wheat|TC103916 Homologue to PIR|S35221|S35221 globulin Beg1 precursor – barley, partial (89%)	100%/1997	0
Hordeum vulgare Barley	Barley|TC87397 PIR|S35221|S35221 globulin Beg1 precursor, complete	77%/1831	1.1e-222
Oryza sativa Rice	Rice|TC148637 GP|4097102|gb|AAD10375.1||U4532 2 globulin-like protein {Oryza sativa}, partial (98%)	70%1542	1.0e-138
Zea mays Maize	Maize|TC193246 PIR|B53234|B53234 vicilin-like storage protein Glb1-L, embryo-maize, partial (98%)	66%/1650	3e-119
Sorghum bicolor Sorghum	Sorghum|TC57088 similar to PIR|B53234|B53234 vicilin-like storage protein Glb1-L, embryo-maize, partial (66%)	70%/1290	5.3e-113
Helianthus annuus Sunflower	Sunflower|TC12996 weakly similar to GP|13183177|gp|AAK15089.1|AF240 006_1|AF240006 7S globulin {Sesamum indicum}, partial (24%)	54%/805	2.9e-13
Secale cereale Rye	s_cereale|BE704590	57%/620	3.9e-05
Glycine max Soybean	Soybean|TC184754 similar to GP|4193320|gp|AAD10139.1|AF0454 73 histone deacetylase {Zea mays}, partial (24%)	58%/346	0.00041
Lycopersicon esculentum Tomato	Tomato|BE458586 weakly similar to SP|P09799|VCLA_ Vicilin GC72-A precursor (alpha-globulin A). [Upland cotton] {Gossypium hirsutum}, partial (13%)	56%/399	0.0024

homologies between WP5212 and known immunomodulatory proteins. It was found that WP5212 shared sequence homologies with the peanut allergen Ara h 1 (accession number P43237; 25% identity across 630 amino acids; Table 5), which is associated with food-induced type 1 hypersensitivity. The antibody-binding epitopes have been mapped for Ara h 1, and WP5212 shared homology with three of four immunodominant epitopes, as well as four of five other commonly recognized epitopes (171). WP5212 also had sequence homology with two other plant allergens, a soybean protein (*Glycine max*, accession number BAB21619) and a dandelion root protein (*Taraxacum officinale*, accession number RAP_TAROF).

It has been suggested that environmental modification of autoimmunity occurs by cross-reactivity, termed molecular mimicry, between environmental antigens and self proteins (158, 172). Immune activation against an offending dietary antigen could lead to immune targeting of an otherwise innocuous self protein and damage to the tissue. A BLAST search of the human genome and NCBI databases retrieved sequence homologies to tight junction protein 2 (*Homo sapiens*, accession number 4759342; *Gallus gallus* (chicken), accession number 7512238), similar to tight junction protein ZO-1 (*H. sapiens*, accession number 17436387) and similar to tight junction protein ZO-2 (*H. sapiens*, accession number 13639591). Tight junction proteins control the permeability of the intestinal epithelium, providing a potential link between the wheat protein WP5212, the gut and diabetes development.

Table 5. Proteins with sequence homologies to WP5212.

Group[a]	Homologous protein[b]	Expect value[c]	% identity/no. amino acids
1	Allergen Ara h 1, clone P17 precursor (Ara h 1) Gi|1168390|sp|P43237|AL11_ARAHY	$4e^{-25}$	25%/630
	Ara h 1 commonly recognized epitope 2[b]	7.7×10^5	60%/5
	Ara h 1 immunodominant epitope 3[b]	7.7×10^5	50%/6
	Ara h 1 immunodominant epitope 4[b]	5.4×10^4	100%/4
	Ara h 1 commonly recognized epitope 8[b]	4.3×10^6	80%/5
	Ara h 1 commonly recognized epitope 12[b]	5.4×10^4	83%/6
	Ara h 1 commonly recognized epitope 13[b]	8.9×10^2	70%/10
	Ara h 1 immunodominant epitope 17[b]	7.7×10^5	80%/5
	(AB046874) allergen Gly m Bd 28K (*Glycine max*) Gi|12697782|dbj|BAB21619.1|	<0.005	21%/483
2	Root allergen protein (RAP), dandelion Gi|7388038|sp|O49065|RAP_TAROF	<688	31%/58
3	Tight junction protein, ZO-2, chicken Gi|7512238|pir||JE0366 37	<17	29%/124
	Similar to tight junction protein ZO-1 (*Homo sapiens*) Gi:17436387	<1.2	28%/142
	Tight junction protein 2 (*Zona occludens* 2); Friedreich ataxia region gene X104 (Tight junction protein ZO-2) (*H. sapiens*) Gi:4759342	<5.8	31%/133
	Similar to Tight junction protein ZO-2 (*Z. occludens* 2 protein) (tight junction protein 2) (*H. sapiens*) Gi:13639591	<5.8	31%/133

[a] Group 1 refers to homologies retrieved with and without the low complexity filter. Group 2 refers to homologies retrieved with the low complexity filter only. Group 3 refers to homologies retrieved without the low complexity filter only.

[b] Homology achieved using the sequence homology matrix PAM-30, recommended for comparing sequences less than 35 amino acids in length.

[c] Expect value is defined as follows by NCBI as a parameter that describes the number of hits one can "expect" to see just by chance when searching a data base of a particular size. The *E* value describes the random background noise that exists for matches between sequences. For example, an *E* value of 1 assigned to a hit can be interpreted as meaning that in a data base of the current size one might expect to see 1 match with a similar score simply by chance.

Study 3: Antibody reactivity to candidate type 1 diabetes-related wheat proteins in the NOD mouse

Wheat protein-based diets can modulate diabetes outcome in NOD mice. Mice fed a non-purified, defined, mainly wheat-based, NTP diet (n = 26) had a final diabetes incidence more than twice that of mice fed the protective HC semi-purified diet (n = 26; 58% vs. 27%, p = 0.01; Figure 15, *inset*). The rate of disease appearance was significantly decreased in mice fed the HC diet in comparison to those fed the NTP diet ($p < 0.05$; Figure 15).

Prediabetic mice have increased frequency and intensity of antibody reactivity to WP5212. To determine if antibody reactivity to WP5212, WP12111, WP23112 and WPCON was related to diabetes risk in the NOD mouse the clones were screened with prospective serum samples from individual 80 d female prediabetic (n = 15; mean age at diabetes onset = 155 ± 41 days), asymptomatic (no clinical symptoms of diabetes by 270 d; n = 11; mean age at end of study = 269 ± 1 days) NOD, and NOR (n = 7; age at necropsy = 70 days) mice fed cereal-based diets (Figure 16). Antibody reactivity was measured by densitometry and is reported as mean intensity/pixel. Antibody reactivity to clones WP12111, WP23112 and WPCON was undetectable in the majority of mice, therefore only data for WP5212 are shown. Representative plaque lifts of WP5212 screened with antibodies from NOD mice are shown in Figure 16 (*panel A*). Antibody reactivity to clone WP5212 in prediabetic mice (63.5 ± 18.5 mean intensity/pixel) tended to be higher than in asymptomatic (48.5 ± 21.2 mean intensity/pixel; ANOVA/LSD, p = 0.02) and diabetes-resistant (31.1 ± 10.1; p = 0.00005) mice (Figure 16, *panel B*). Asymptomatic NOD mice also had increased antibody reactivity to WP5212 compared

Figure 15. Modification of diabetes development in non-obese diabetic (NOD) mice by wheat-based diets. Final diabetes incidence (*inset*) and survival curves in NOD mice fed from weaning a mixed, cereal-based (mainly wheat-based) NTP diet, or the semipurified diet in which the sole amino acid source was HC plus supplemental sulfur amino acids. The rate of disease development by survival analysis was significantly delayed in mice fed the HC diet (log-rank/Kaplan-Meier test; $p < 0.05$). *Inset*, animals fed the NTP diet had the highest incidence, 58% ($n = 26$/group, Fisher's exact (two-tailed) test, $p = 0.01$ vs. HC 27%).

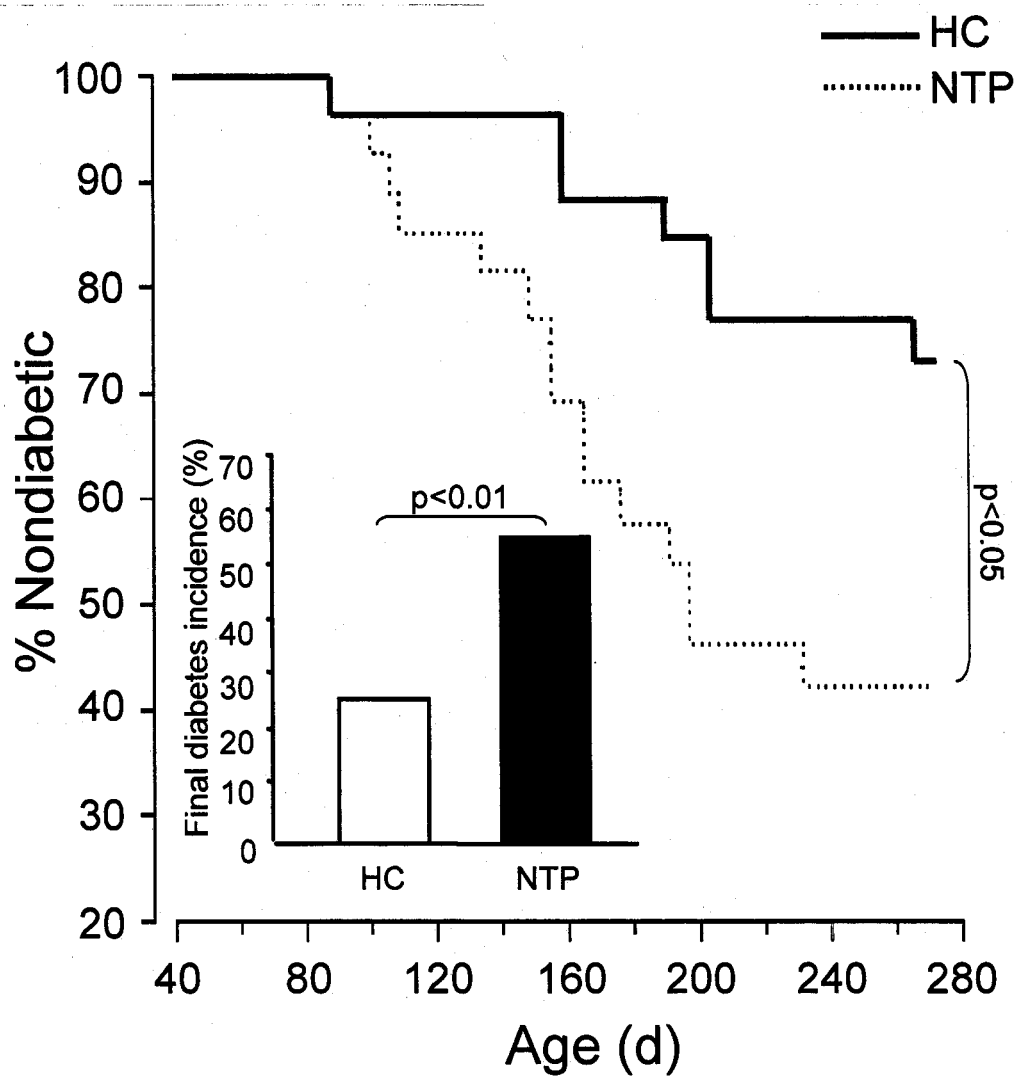
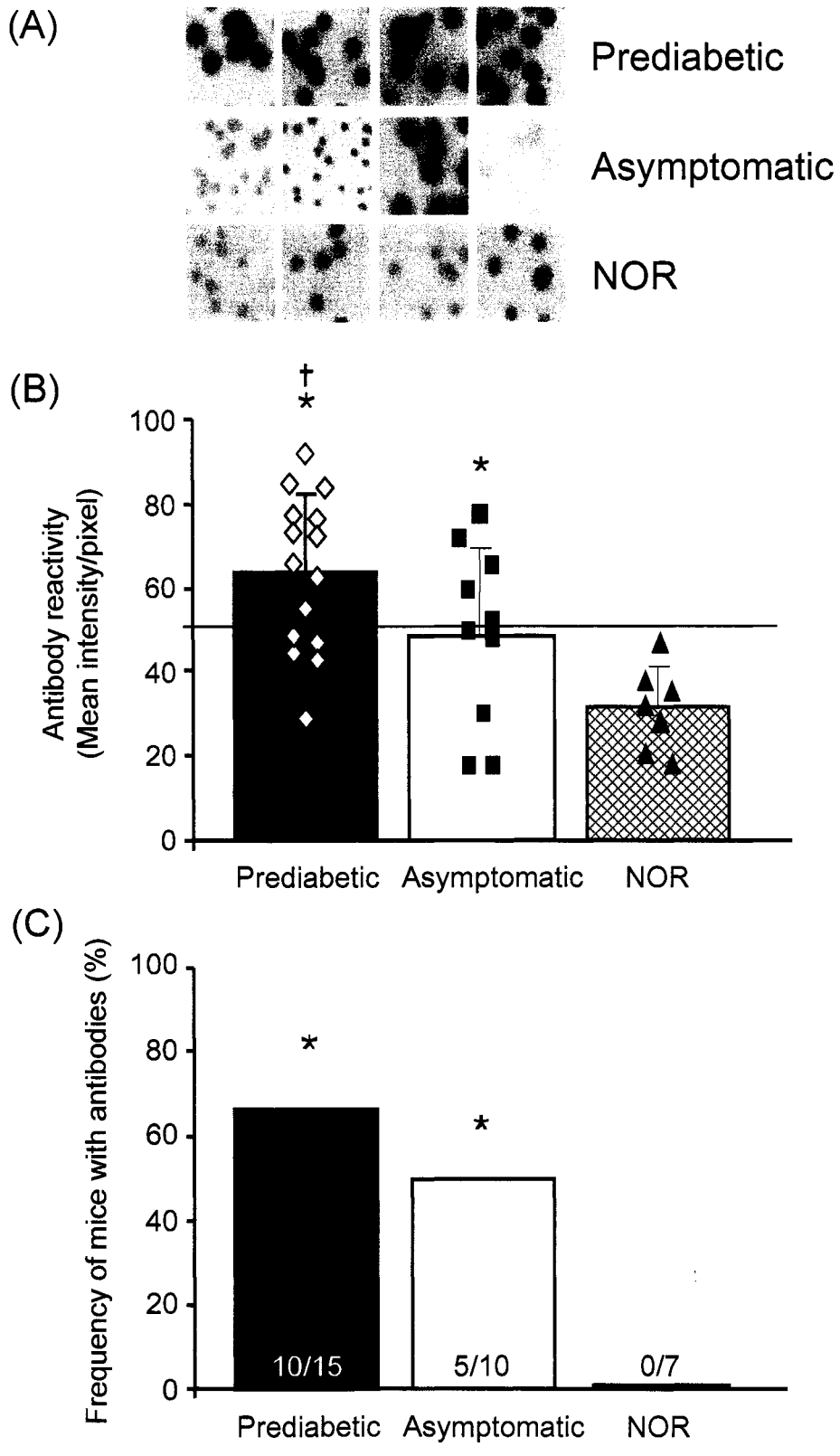

HC
NTP
100
90
80
70
60
50
40
30
20
% Nondiabetic
p<0.05
Final diabetes incidence (%)
70
60
50
40
30
20
10
0
p<0.01
HC
NTP
40
80
120
160
200
240
280
Age (d)

Figure 16. WP5212 screened with serum antibodies from prediabetic, asymptomatic, or control NOD mice. *Panel A*, plaque lifts of clone WP5212 screened with antibodies from four representative prediabetic, asymptomatic or NOR mice. *Panel B*, mean antibody reactivity (mean intensity/pixel) ± SD to WP5212 screened with prediabetic NOD (n = 15, black bar), asymptomatic NOD (n = 10, open bar) or NOR (n = 7, cross-hatched bar) mice are shown. Individual values for prediabetic (diamonds), asymptomatic (squares) or control (triangles) mice are shown. ANOVA/LSD; † indicates significant difference vs. asymptomatic mice, $p = 0.02$; * indicates significant vs. NOR mice, $p \leq 0.03$. *Panel C*, the frequency of prediabetic, asymptomatic, or NOR mice with positive antibody reactivity to clone WP5212 is shown. A positive antibody level was defined as an antibody reactivity greater than the mean intensity plus 2 SD (51.2, indicated by red line) of WP5212 screened with NOR mouse serum. Fisher's exact (two-tailed) test; * indicates significant vs. diabetes-resistant NOR mice, $p \leq 0.04$.


(A)
Prediabetic
Asymptomatic
NOR
(B)
†
*
*
100
80
60
40
20
0
Antibody reactivity
(Mean intensity/pixel)
Prediabetic
Asymptomatic
NOR
(C)
*
*
100
80
60
40
20
0
Frequency of mice with antibodies (%)
10/15
5/10
0/7
Prediabetic
Asymptomatic
NOR

with control NOR mice ($p = 0.03$).

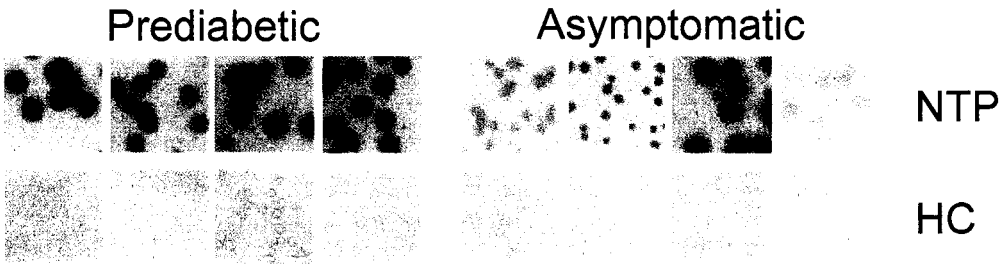
The frequency of mice that developed antibodies to WP5212 was determined (Figure 16, *panel C*). A positive antibody level was defined as an antibody reactivity value greater than the mean intensity plus 2 SD for WP5212 screened with NOR serum (mean intensity/pixel + 2 SD = 51.2). More 80 d prediabetic (66.7%; $p = 0.005$) and asymptomatic (50%; $p = 0.04$) mice had antibodies to WP5212 than resistant mice (0%), but there was no difference in frequency of WP5212 antibody development between diabetic and asymptomatic mice.

Antibody development to WP5212 is diet induced. To examine whether development of antibodies to WP5212 is specific to the wheat-based NTP diet, clone WP5212 was screened with serum from prediabetic and asymptomatic mice fed the protective HC diet. Examples of WP5212 plaque lifts are shown in Figure 17 (*panel A*). Prediabetic mice fed the NTP diet (63.5 ± 18.5 mean intensity/pixel) had significantly higher antibody reactivity than prediabetic (5.4 ± 4.4 mean intensity/pixel) and asymptomatic (6.1 ± 4.6 mean intensity/pixel) animals fed the HC diet ($p < 10^{-6}$; Figure 17, *panel B*). Asymptomatic mice fed the NTP diet (48.5 ± 21.2 mean intensity/pixel) also had significantly higher antibody reactivity than the HC-fed prediabetic and asymptomatic mice ($p < 10^{-5}$; Figure 17, *panel B*).

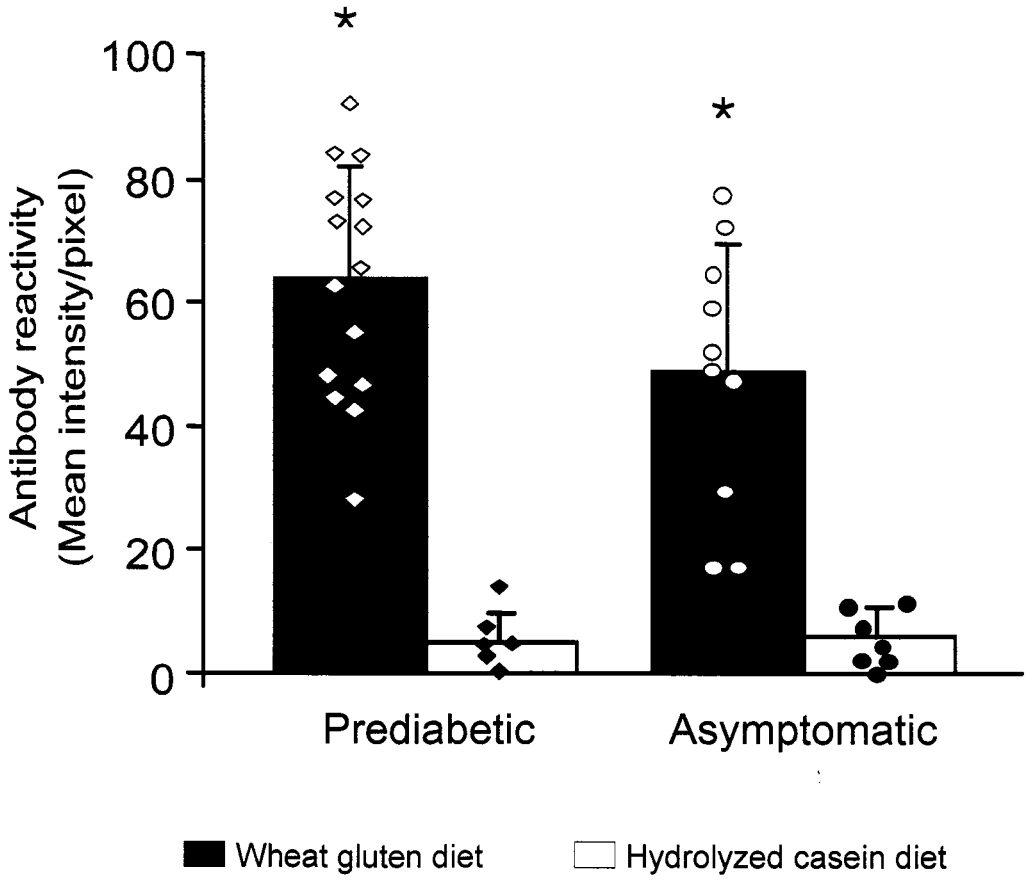
NOD.Itgb7$^{-/-}$ mice have decreased WP5212 antibodies in comparison with prediabetic NOD mice. Our lab has proposed that dietary antigens, including WP5212, trigger immune activation in the gut associated lymphoid tissues (GALT). The integrin $\alpha4\beta7$ allows lymphocytes to traffic to the GALT and acts as a marker of gut associated

Figure 17. NOD mice fed a wheat-free diet do not develop antibodies to WP5212. *Panel A*, plaque lifts of clone WP5212 screened with antibodies from four representative prediabetic and asymptomatic mice fed either a wheat-based NTP or protective HC diet. *Panel B*, mean antibody reactivity (mean intensity/pixel) ± SD to WP5212 screened with prediabetic NOD (n = 15) or asymptomatic NOD (n = 10) mice fed the NTP diet (open bars) or prediabetic NOD (n = 6) or asymptomatic NOD (n = 7) mice fed the HC diet (filled bars) are shown. ANOVA/LSD; * indicates significant vs. NOD mice fed the HC diet, $p \leq 10^{-5}$).

(A)


Prediabetic
Asymptomatic
NTP
HC

(B)


Antibody reactivity
(Mean intensity/pixel)
100
80
60
40
20
0
*
*
Prediabetic
Asymptomatic
Wheat gluten diet
Hydrolyzed casein diet

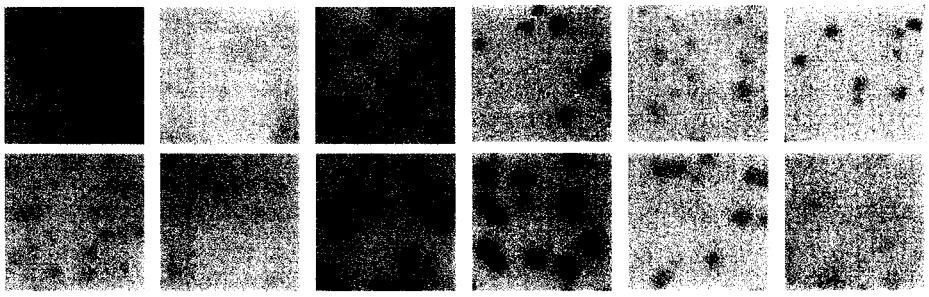
lymphocytes (173-177). Therefore, it was hypothesized that genetically susceptible NOD mice that lack the β7 (Itgb7) integrin will not develop diabetes or antibodies to WP5212 due to a lack of stimulation of the GALT by dietary antigens. Dr. Ed Leiter (Jackson Laboratories, Bar Harbor ME) developed NOD mice lacking L-selectin (L-Sel), β7 integrin or both genes. Female NOD.Itgb7$^{-/-}$ mice demonstrate delayed onset of diabetes and an overall decrease of disease incidence (approximately 35% incidence vs. 95% in female NOD mice; E. Leiter, unpublished data). These knockout mice have significantly decreased lymphocyte populations in intestinal Peyer's patches. The NOD.Itgb7$^{-/-}$/L-Sel$^{-/-}$ mice also show delayed diabetes onset and a decrease in diabetes incidence (approximately 35% incidence vs. 95% in female NOD mice; E. Leiter, unpublished data). The NOD.L-Sel$^{-/-}$ mice have a minor decrease in diabetes incidence compared to NOD mice (approximately 85% incidence vs. 95% in female NOD mice; E. Leiter, unpublished data). Dr. Leiter kindly donated serum samples from NOD.Itgb7$^{-/-}$ and NOD.Itgb7$^{-/-}$/L-Sel$^{-/-}$ mice to study the dependence of WP5212 antibody development on β7 integrin.

We screened WP5212 plaque lifts with serum IgG antibodies from 70 d NOD.Itgb7$^{-/-}$ mice (n = 12) and NOD.Itgb7$^{-/-}$/L-Sel$^{-/-}$ double knockout mice (n = 8). The plaque lifts from NOD.Itgb7$^{-/-}$ mice are shown in Figure 18 (*panel A*). NOD.Itgb7$^{-/-}$ mice (22.2 ± 13.6 mean intensity/pixel) had decreased antibody reactivity to WP5212 compared with both prediabetic (63.5 ± 18.5 mean intensity/pixel) and asymptomatic (48.5 ± 13.6 mean intensity/pixel) NOD mice ($p < 10^{-6}$ and $p = 0.0005$, respectively; Figure 18, *panel B*). Interestingly, the NOD.Itgb7$^{-/-}$ mice had decreased WP5212 antibody reactivity compared with NOD.Itgb7$^{-/-}$/L-Sel$^{-/-}$ double knockout mice

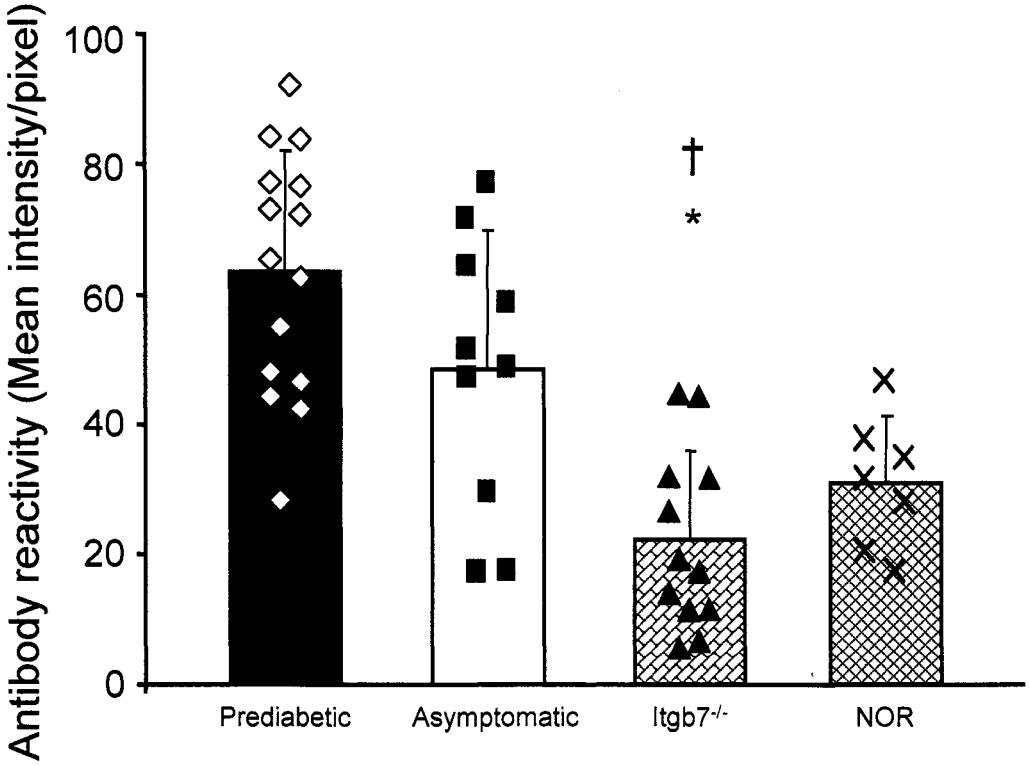
Figure 18. Antibody reactivity to WP5212 in NOD.Itgb7$^{-/-}$ mice. *Panels A*, plaque lifts of clone WP5212 screened with antibodies from NOD.Itgb7$^{-/-}$ mice (n = 12). *Panel B*, mean antibody reactivity (mean intensity/pixel) ± SD to clone WP5212 screened with prediabetic NOD (diamonds), asymptomatic NOD (squares), NOD.Itgb7$^{-/-}$ (triangles) or NOR (crosses) mice is shown. ANOVA/LSD; † denotes $p \leq 0.03$ vs. prediabetic NOD mice; * denotes $p = 0.0005$ vs. asymptomatic NOD mice.

(A)

NOD.Itgb7$^{-/-}$



(B)


Antibody reactivity (Mean intensity/pixel)
100
80
60
40
20
0
†
*
Prediabetic
Asymptomatic
Itgb7-/-
NOR

(47.7 ± 12.6 mean intensity/pixel; $p = 0.001$; data not shown). NOD.Itgb7$^{-/-}$/L-Sel$^{-/-}$ mice had decreased antibody reactivity relative to prediabetic ($p = 0.03$) but not asymptomatic NOD mice (data not shown). The WP5212 antibody reactivity levels for both knockout mouse strains were not different from control NOR mice. However, in order to evaluate properly the data obtained for the NOD.Itgb7$^{-/-}$/L-Sel$^{-/-}$ mice, the appropriate control mouse strain, NOD.L-sel$^{-/-}$, must also be considered. We have requested serum samples from L-Sel$^{-/-}$ mice from Dr. Leiter in order to follow up this study.

Study 4: Antibody reactivity to WP5212 in a patient with type 1 diabetes and celiac disease

Case report: A highly wheat sensitive patient with both type 1 diabetes and celiac disease. A 28 year old Caucasian female with a 10 year history of T1D presented with persistent diarrhea, which was followed by oral ulcerations and severe lip swelling (Figure 19). The patient was having 4 to 10 bowels movements in a 24 h period, including nocturnal stools. There was no family history of bowel disease or any joint, skin, or eye symptoms. Colonoscopy and small bowel follow through were normal. Biopsies of the oral ulceration determined them to be non-caseating granulomas. The granulomas were negative for IgG, IgA, IgM, C3, fibrinogen and albumin. Special stains for tuberculosis, fungus and other infectious agents were negative as well.

The diarrhea and lip swelling improved with the initiation of a standard gluten-free diet. However, the symptoms recurred within a few months with worsening of the oral ulcerations, lip swelling and continued diarrhea. Anti-gliadin IgG (31 EU/ml) and endomysial antibody (titer 5) levels were positive. Celiac disease was confirmed with a

Figure 19. Oral inflammation and fistula in a highly wheat sensitive patient with both type 1 diabetes and celiac disease. *Panel A*, the patient displayed swelling of the lips and could only open her mouth 1.5 cm (as pictured). *Panel B*, an ulcerating fistula formed through the mucosa of the patient's lower lip.

(A)

(B)

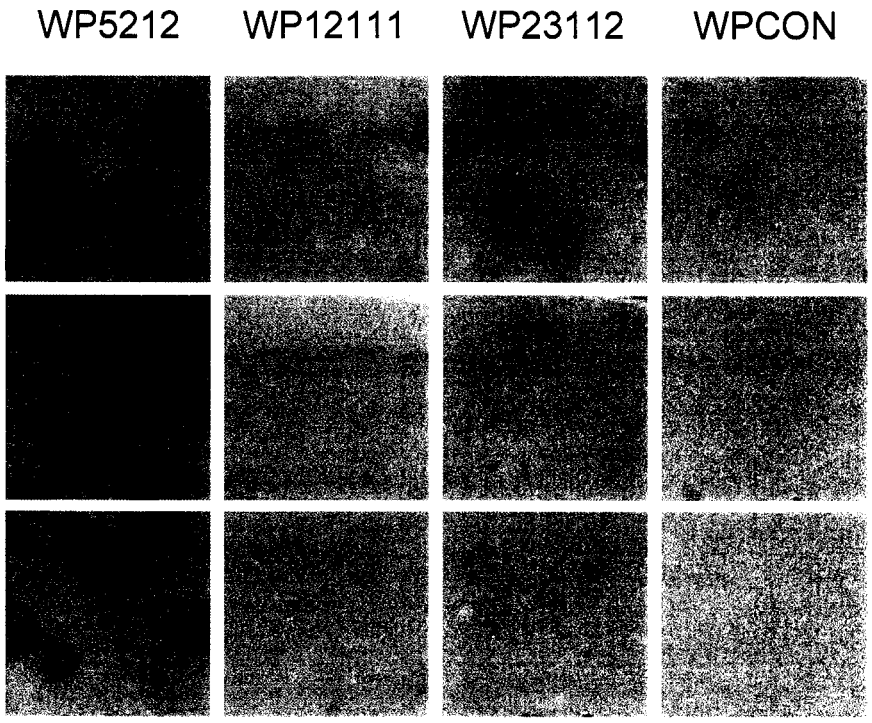
duodenal biopsy showing mild villous atrophy despite 6 months on a standard gluten-free diet.

After approximately two years on a gluten-free diet, the mucosa of the patient's lower lip was extensively ulcerated and a fistula formed through the lower right lip area (Figure 19, *panel A* and *B*). The patient could only open her mouth 1.5 cm wide (Figure 19, *panel A*). Both lower and upper lips were swollen. The patient was having 7-10 bowel movements in a 24 h period with nocturnal incontinence. A magnetic resonance scan of her head and neck area showed no areas of fibrosis, therefore a diagnosis of Melkersson-Rosenthal was less likely. Following this episode, the patient initiated a specific-carbohydrate and cereal-free diet, as described in the book "Breaking the Vicious Cycle: Intestinal Health Through Diet" by E. Gottschall (178). Within a few weeks, the diarrhea decreased with the patient having an average of 2 bowel movements per day. The oral ulcerations and swelling were also diminished. Over a period of a few months, the patient's lips returned to normal with a scar and fibrosis in the area of the former fistula.

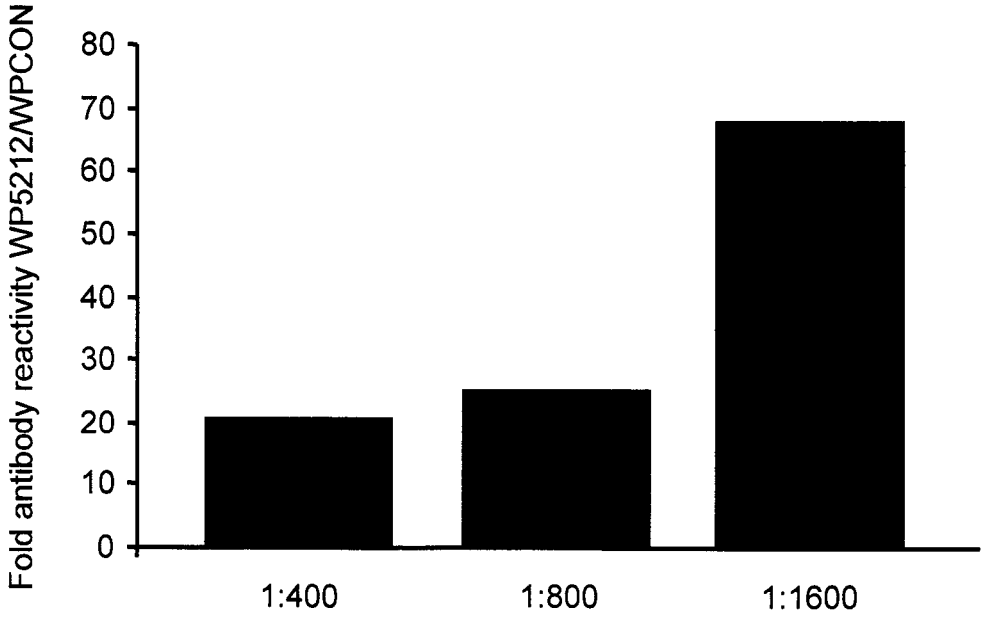
High titer WP5212 antibodies in a patient with both type 1 diabetes and celiac disease. To determine whether antibody reactivity to WP5212 was increased in the T1D/CD patient, WP5212, as well as WP12111, WP23112 and WPCON were screened with serial dilutions of sera from the patient and from healthy control subjects (n = 8). Antibody reactivity was normalized relative to the negative control, WPCON, for all clones. The specificity of antibody reactivity to WP5212 increased upon dilution of the serum (1:400<1:800<1:1600; Figure 20, *panel A* and *B*) while that of control subjects remained consistently low to undetectable (data not shown). The patient had increased

Figure 20. Serial dilution of serum antibodies from a patient with type 1 diabetes and celiac disease. *Panel A*, increasing dilutions (1:400, 1:800 and 1:1600 in blocking buffer) of the patient's serum were used to screen the four wheat clones. *Panel B*, relative antibody reactivity to WP5212 (WP5212/WPCON) as determined by densitometric analysis of plaque lifts screened with serial dilutions of the patient's serum.

(A)


WP5212
WP12111
WP23112
WPCON

(B)


Fold antibody reactivity WP5212/WPCON
80
70
60
50
40
30
20
10
0
1:400
1:800
1:1600

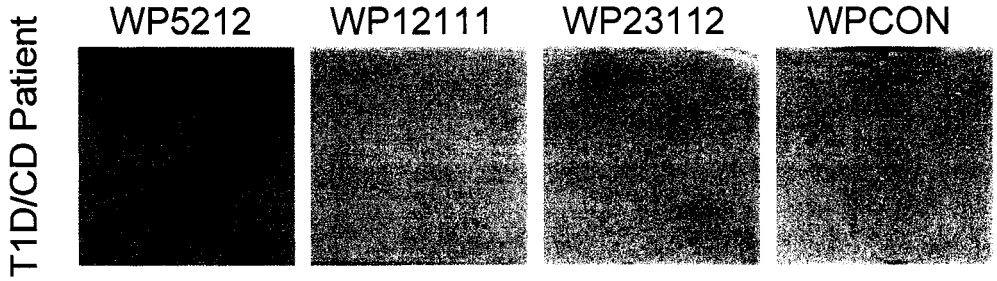
antibody reactivity to WP5212 in comparison to healthy controls (Figure 21, *panel A* and *B*). Antibody reactivity to WP12111 and WP23112 was low at all dilutions for both the patient and the healthy controls (Figure 20 and 21).

Study 5: Antibody reactivity to WP5212 in patients with type 1 diabetes

Expression of WP5212 in High-Five™ and Sf21 insect cells. Expression of the full length WP5212 protein has proven difficult. The protein is toxic to *E. coli* resulting in low plasmid yields, and consequently low protein yields (see Appendix I). We sent the WP5212 pBK-CMV phagemid to Dr. Markus Walz in the lab of our collaborator Dr. Hubert Kolb (German Diabetes Research Institute, Heinrich Heine University of Düsseldorf, Germany) for construction of a WP5212 recombinant baculovirus. High-Five™ (Invitrogen) insect cells were infected with the recombinant WP5212 baculovirus. The insect cells were lysed in PBS and the lysate was sent to our lab. To verify WP5212 protein expression, 1D SDS-PAGE and Western blotting were performed. The expected molecular weight of the WP5212 protein is 66 kDa. Coomassie staining of the 1D SDS-PAGE gel confirms over-expression of an approximately 61 kDa protein in the WP5212 baculovirus infected insect cells (Figure 22, *panel A*). The Western blot was probed with serum from a WP5212 antibody positive diabetic BB rat (confirmed by the experiment in Study 2). The 61 kDa protein was bound by antibodies from the rat supporting the identity of this protein as WP5212 (Figure 22, *panel B*). There was weak antibody binding to other proteins in the uninfected or wild type virus infected insect cells, but none in the 61 kDa region. The WP5212-baculovirus infected insect cell lysate provided by Markus Walz was used to infect Sf21 insect cells. Viral plaques were picked for the

Figure 21. A patient with type 1 diabetes and celiac disease has increased antibody reactivity to the wheat protein WP5212 compared with healthy control subjects. *Panel A*, plaque lifts of clones WP5212, WP12111, WP23112, and WPCON screened with serum diluted 1:800 in blocking buffer from a patient with type 1 diabetes and celiac disease. *Panel B*, relative antibody reactivity (relative to antibody reactivity to WPCON) to the recombinant wheat proteins screened with healthy control serum ($n = 8$, open bars, mean ± SD) or patient serum (n = 1, black bars).

(A)


WP5212
WP12111
WP23112
WPCON
T1D/CD Patient

(B)

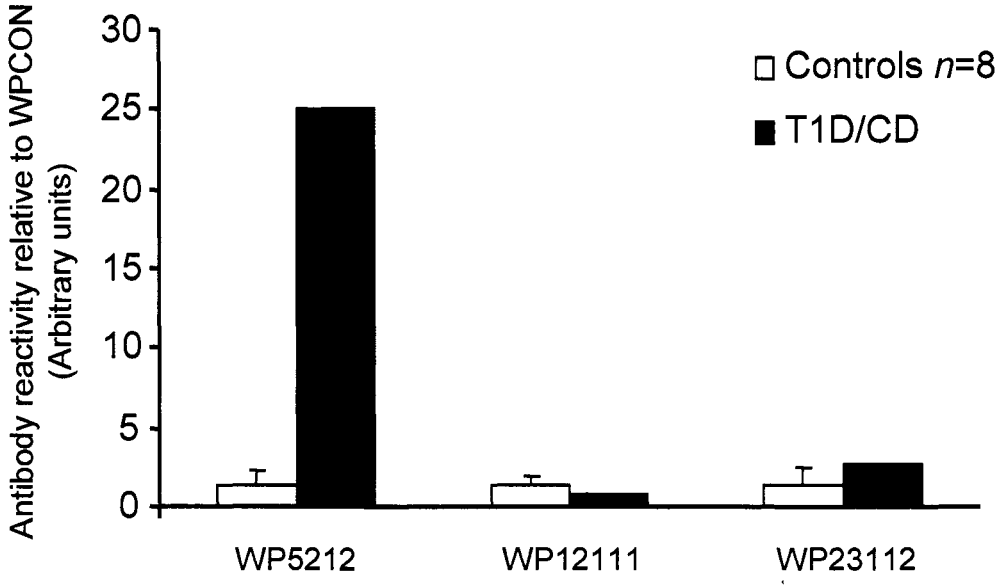
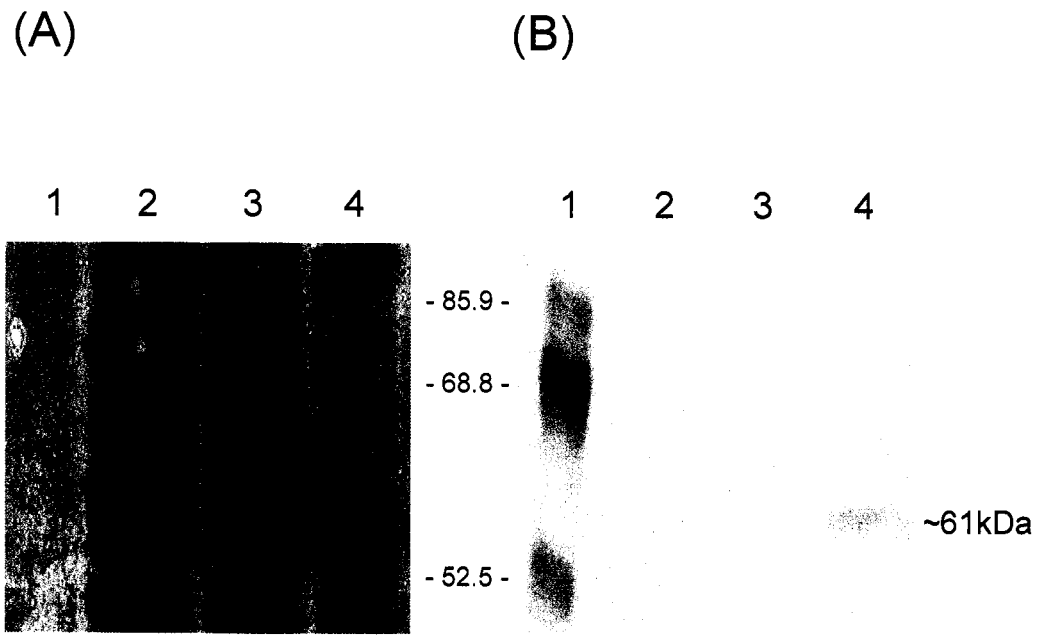

Antibody reactivity relative to WPCON
(Arbitrary units)
30
25
20
15
10
5
0
Controls n=8
T1D/CD
WP5212
WP12111
WP23112

Figure 22. Western blot analysis of WP5212-baculovirus infected insect cell lysate using serum from a WP5212 antibody positive diabetic BB rat. Insect cell lysate samples were obtained from Drs. Markus Walz and Hubert Kolb (Düsseldorf, Germany). *Panel A*, WP5212 protein expression in insect cells was detected by Coomassie staining a 1D SDS-PAGE gel of insect cell lysate infected with recombinant baculovirus (lane 1, Benchmark prestained ladder; lane 2, control BacPAK6 infected High-Five™ insect cell lysate; lane 3, uninfected High- Five™ insect cell lysate; lane 4, WP5212-baculovirus infected High- Five™ insect cell lysate). *Panel B*, diabetic BB rat serum known to be WP5212 antibody positive was used to probe the Western blot of recombinant baculovirus infected insect cell lysate to determine expression of WP5212 (lane 1, Benchmark prestained ladder; lane 2, control BacPAK6 infected High-Five™ insect cell lysate; lane 3, uninfected High-Five™ insect cell lysate; lane 4, WP5212-baculovirus infected High-Five™ insect cell lysate).


(A)
(B)
1 2 3 4
1 2 3 4
- 85.9 -
- 68.8 -
- 52.5 -
~61kDa

production of clonal viruses. Sf21 cells were infected with the selected plaques to produce primary viral stocks. The insect cells used to produce the passage one viral stocks were lysed in PBS and used for 1D SDS-PAGE and Western blotting. Coomassie staining of the 1D SDS-PAGE gel identifies an approximately 63 kDa over-expressed protein in WP5212 baculovirus clones 1, 2, and 4 to 7 (Figure 23, *panel A*). This protein was not observed in uninfected or wild type infected insect cells. The Western blot was probed with serum from the case study patient who had high titer antibodies to WP5212. The 63 kDa protein from WP5212 baculovirus clones 1, 2, and 4 to 7 was bound by antibodies from the patient again strongly supporting the identity of this protein as WP5212 (Figure 23, *panel B*). The patient did not have antibodies to proteins from uninfected or wild type virus infected insect cells.

To confirm the identity of the antibody bound protein, WP5212 polyclonal antiserum was produced in rabbits. Two rabbits were co-immunized with two WP5212-specific peptides. Pre-immune rabbit serum samples do not have WP5212 antibodies (Figure 24, *panel A*). The immunized rabbits developed WP5212 specific antibodies (Figure 24, *panel A*). WP5212 expressed in insect cells appears as a doublet (Figure 24, *panel B*).

Patients with type 1 diabetes have increased antibody reactivity to WP5212. The study group consisted of patients with type 1 diabetes (n = 22; mean age = 23.3 ± 7.1 years; mean age at T1D diagnosis = 11 ± 8.4; Table 6), celiac disease (n = 3; mean age = 28.3 ± 4.2 years), both diseases (n = 3; mean age = 27.7 ± 15.5 years; mean age at T1D diagnosis = 15 ± 4.2 years) or healthy control subjects (n = 14; mean age = 27.2 ± 5.7

Figure 23. Western blot analysis of WP5212-baculovirus infected insect cell lysate using serum antibodies from a patient with type 1 diabetes and celiac disease. *Panel A*, recombinant baculovirus clones were evaluated for WP5212 protein expression by Coomassie staining a 1D SDS-PAGE gel of insect cell lysate samples. *Panel B*, serum IgG antibodies from a patient with type 1 diabetes and celiac disease known to be WP5212 antibody positive was used to probe the Western blot of WP5212-baculovirus infected insect cell lysate samples to determine expression of the protein. (lane 1, Benchmark prestained ladder; lane 2, uninfected insect cell lysate; lane 3, AcMNPV wild type baculovirus infected insect cell lysate; lane 4, BacPAK6 control vector baculovirus infected insect cell lysate; lanes 5 to 11, clones 1-7 recombinant WP5212-baculovirus infected insect cell lysate samples)

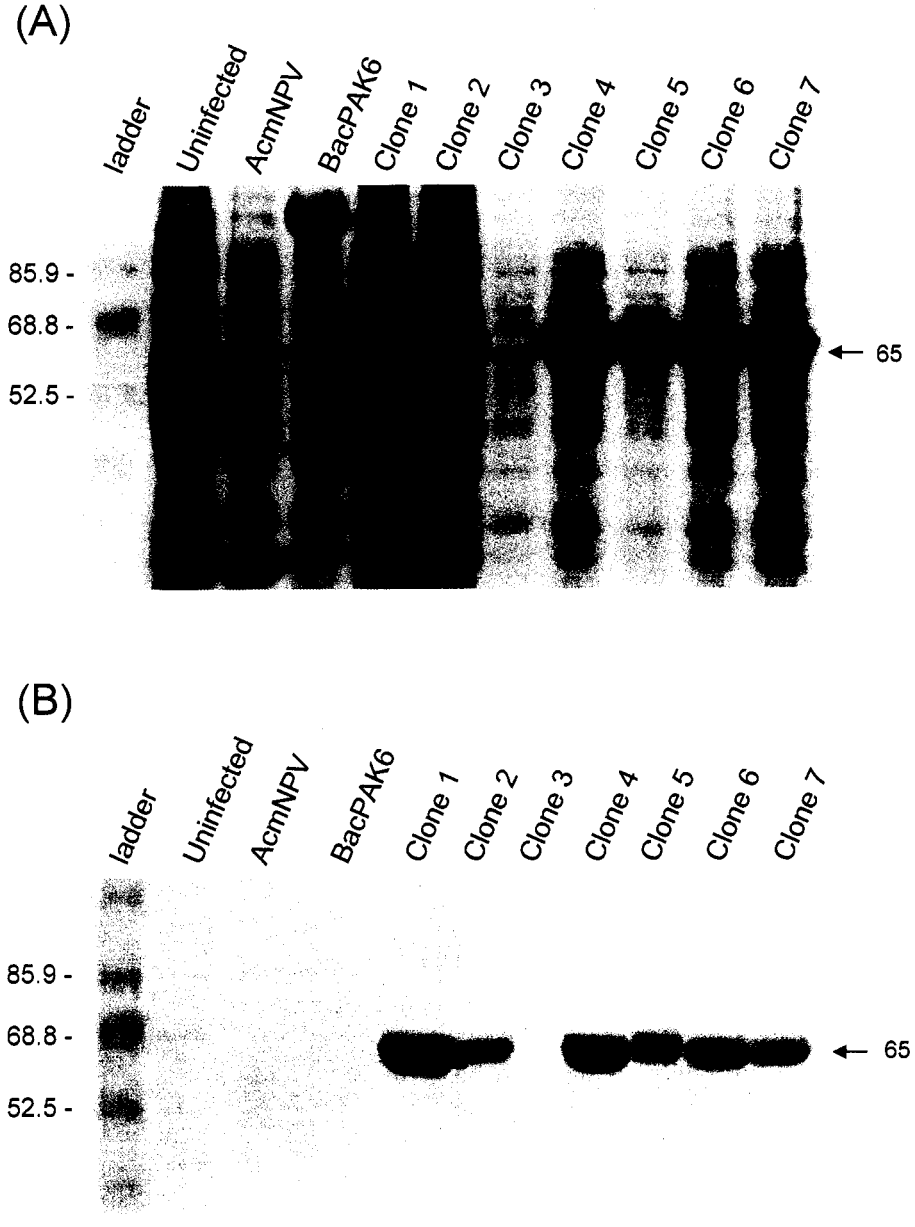
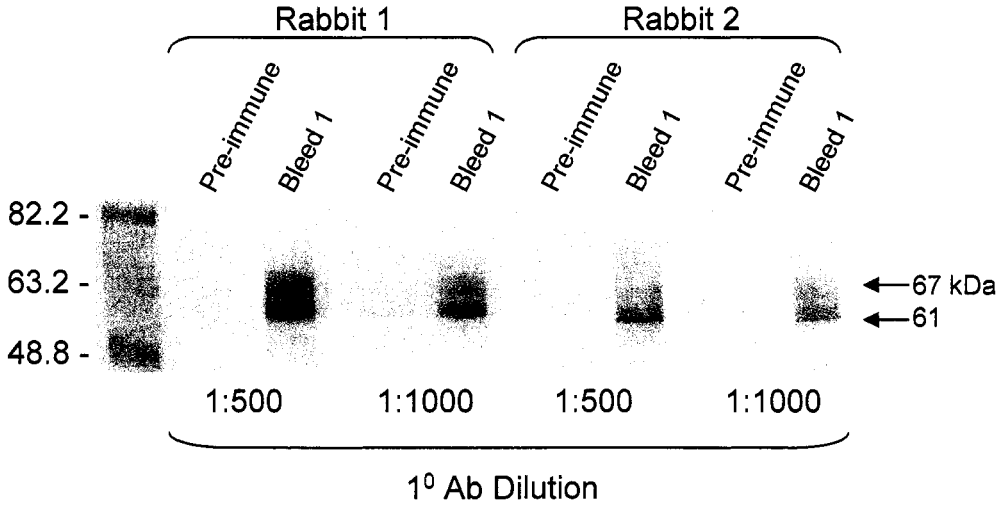

(A)
ladder
Uninfected
AcmNPV
BacPAK6
Clone 1
Clone 2
Clone 3
Clone 4
Clone 5
Clone 6
Clone 7
85.9 -
68.8 -
52.5 -
65
(B)
ladder
Uninfected
AcmNPV
BacPAK6
Clone 1
Clone 2
Clone 3
Clone 4
Clone 5
Clone 6
Clone 7
85.9 -
68.8 -
52.5 -
65

Figure 24. Confirmation of WP5212 expression in Sf21 insect cells by probing Western blots with polyclonal WP5212-specific antibodies. *Panel A*, 1D Western blots mounted onto a Hoefer decaprobe for screening with pre- or post-immune serum diluted 1:500 or 1:1000 in blocking buffer from two rabbits immunized with WP5212 specific peptides. Pre-immune serum does not bind the WP5212 protein. *Panel B*, enlargement of the region in which WP5212 is expressed. WP5212 is expressed as a doublet in Sf21 insect cells and appears as 61 and 67 kDa isoforms.

(A)


Rabbit 1
Rabbit 2
Pre-immune
Bleed 1
Pre-immune
Bleed 1
Pre-immune
Bleed 1
Pre-immune
Bleed 1
82.2 -
63.2 -
48.8 -
67 kDa
61
1:500
1:1000
1:500
1:1000
1⁰ Ab Dilution

(B)

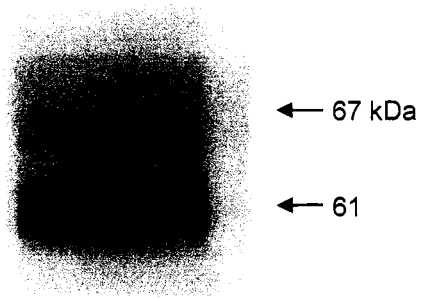

67 kDa
61

Table 6. Characteristics of the human study groups

Subject type	N	Sex ratio (M/F)[a]	Mean age ± SD (years)[a]	Mean age ± SD at T1D diagnosis (years)[a]
T1D	22	10/12	23.3 ± 7.1	11 ± 8.4[b]
CD	3	1/2	28.3 ± 4.2	N/A
T1D/CD	3	0/3	27.7 ± 15.5	15 ± 4.2[b]
Control	14	5/9	27.2 ± 5.7	N/A

[a] No significant differences in sex ratio, age or age at T1D diagnosis among the groups.
[b] Data for one patient missing
N/A: not applicable

years). The subjects participating in the study provided blood samples after giving informed consent. There were no significant differences in age at sampling, sex ratio or age at T1D diagnosis (where applicable) among the groups. The individual characteristics of the study subjects can be found in Table 7. The HLA haplotypes for all diabetic patients, two patients with both diseases and four control subjects were determined (Table 7). Twenty-one out of 22 type 1 diabetes patients and 14 out of 14 control subjects were negative for antibodies to the celiac autoantigen tissue transglutaminase (tTG). Two patients with both T1D and CD were positive for tTG antibody titer and none of the CD patients were positive.

1D SDS-PAGE and Western blotting of WP5212-baculovirus infected insect cell lysate was performed to determine whether patients had increased antibody reactivity to WP5212 in comparison with healthy control subjects. Study subject serum diluted 1:200 was used to screen Western blots of uninfected, wild type virus infected or WP5212-baculovirus infected insect cells (Figure 25, *panels A, B, D, E*). No non-specific binding was observed from the secondary antibody (Figure 25, *panel*). It was found by densitometric analysis that the mean antibody reactivity to WP5212 of the diabetic patients was significantly increased in comparison with control subjects ($p = 0.01$; Figure 26). There was no difference in WP5212 antibody reactivity between patients with CD and diabetes or CD and control subjects (Figure 27). Interestingly, patients with both diabetes and celiac disease showed increased antibody reactivity to WP5212 in comparison to both healthy control subjects and patients with diabetes only ($p \leq 0.000001$; Figure 27). In fact, all three T1D/CD patients had higher mean intensity/pixel values than any of the patients with diabetes alone.

Table 7. HLA haplotype and tissue transglutaminase autoantibody status of individual patients with type 1 diabetes, celiac disease, both diseases and healthy control subjects

Disease status	Subject ID	Sex (M/F)	Age (Y)	DR and DQ HLA Haplotype	tTG level[a]
T1D	D1	F	23	DRB1*03,*13, DR52, DQB1*02,*06	3
	D2	F	20	DRB1*01,*04, DR53, DQB1*03,*05	<1
	D3	F	38	DRB1*01,*03, DR52, DQB1*02,*05	<1
	D4	F	19	DRB1*03,*04, DR52,DR53, DQB1*02,*03	<1
	D5	M	22	DRB1*04,DR53,DQB1*03	<1
	D6	M	21	DRB1*04,DR53,DQB1*03	<1
	D7	M	29	DRB1*04,DR53,DQB1*03(DQ7),*03(DQ8)	<1
	D8	F	8	DRB1*0301,*0401,DR52,DR53,DQB1*0201/*0302	<1
	D9	F	8	DRB1*03,*04, DR53, DQB1*03	<1
	D10	M	22	DRB1*03(DR17),*04,DR52,DR53,DQB1*02,*03	<1
	D11	F	22	DRB1*03(DR17),DR52,DQB1*02	<1
	D12	M	21	DRB1*01,*04,DR53,DQB1*05,*03(DQ7)	<1
	D13	F	27	DRB1*15,*04,DR53,DR51,DQB1*06,*03(DQ7)	<1
	D14	F	26	DRB1*04,*13,DR52,DR53,DQB1*06,*03(DQ8)	<1
	D15	M	25	DRB1*03,*12,DR52,DQB1*02,*03(DQ7)	<1
	D16	F	18	DRB1*03, *12,DR52,DQB1*02,*03(DQ7)	<1
	D17	M	19	DRB1*15,DR51,DQB1*06	
	D18	F	33	DRB1*04*13,DR522,DR53,DQB1*06,*03(DQ7)	<1
	D19	M	30	DRB1*03,*04,DR52,DR52,DQB1*02,*0305	<1
	D20	M	23	DRB1*03,*13,DR52,DQB1*06,*02	<1
	D21	M	33	DRB1*03,*04,DR52,DR53,DQB1*02,*0305	27
	D22	F	25	DRB1*15,*13,DR52,DR51,DQB1*06	<1
CD	CD1	F	27	DRB1*03,*07,DR52,DR53,DQB1*02	<1
	CD2	M	33	DRB1*01,*13,DR52,DQB1*05,*06	6
	CD3	F	25	DRB1*15,*03,DR52,DR52,DR51,DQB1*06,*02	<1
T1D/CD	D/CD1	F	28	DRB1*0301,*0401,DR52,DR53,DQB1*0201,*0302	7
	D/CD2	F	12	DRB1*0101,*0301,DR52,DQB1*0501,*0201	16
	D/CD3	F	43	DRB1*03,*04,DR52,DR53,DQB1*02,*03	>100
Control	C1	F	28	DRB1*03(DR17)*12,DR52,DQB1*02*03	<1
	C2	F	26	DRB1*04,*08,DR53,DQB1*03,*04	<1
	C3	F	38		<1
	C4	F	22	DRB1*13,*15,DR51,DR52,DQB1*06	<1
	C5	M	23	DRB1*03,*07,DR52,DR53,DQB1*02	<1
	C6	M	24		<1
	C7	M	29	DRB1*15,*04,DR53,DR51,DQB1*06,*03(DQ7)	<1
	C8	M	28	DRB1*07,DR53,DQB1*02,*03(DQ9)	<1
	C9	F	21	DRB1*13,*15,DR51,DR52,DQB1*06	<1
	C10	F	22	DRB1*03,*15,DR52,DR53,DQB1*02,*06	<1
	C11	F	29	DRB1*11,*12,DR52,DQB1*03	<1
	C12	F	40	DRB1*11,*14,DR52,DQB1*03(DQ7),*05	<1
	C13	M	28	DRB1*01,*07,DR53,DQB1*05,*02	<1
	C14	F	23	DRB1*15,*07,DR53,DR51,DQB1*06,*03(DQ9)	<1

[a] A tissue transglutaminase autoantibody value less than 4 is negative, a value between 4 and 10 is weakly positive, a value greater than 10 is positive.

Figure 25. Western blot analysis of antibody reactivity to WP5212 screened with serum antibodies from healthy control subjects, patients with type 1 diabetes, celiac disease or both diseases. 1D SDS-PAGE and Western blots of recombinant baculovirus infected insect cell lysate (10 μg/lane) were screened with serum antibodies from healthy control subjects (*panel A*), patients with type 1 diabetes (*panel B*), celiac disease (*panel D*) or both diseases (*panel E*). Non-specific binding from the secondary antibody was also tested (*panel C*). Red markers indicate WP5212 antibody positive individuals.

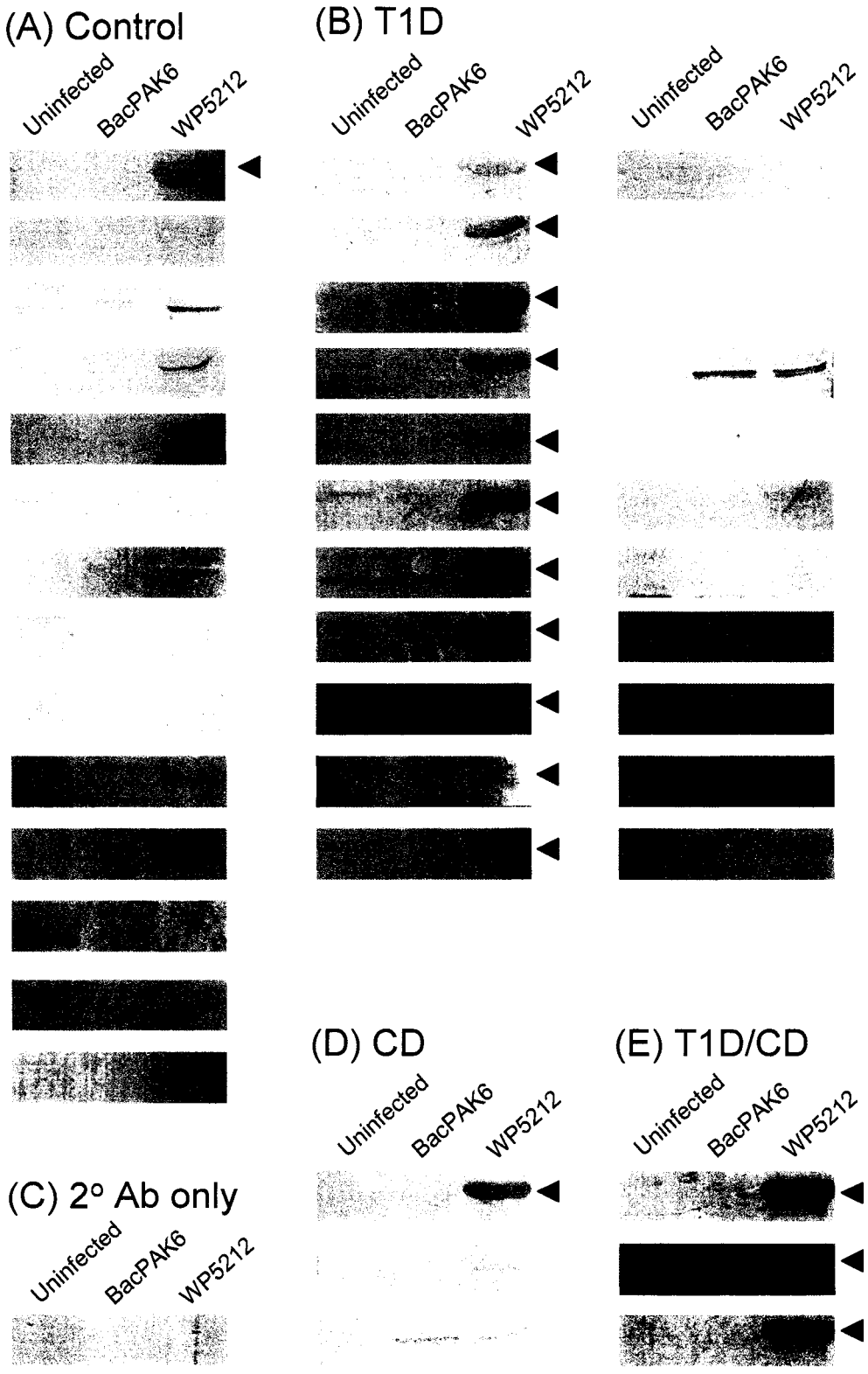

(A) Control
Uninfected
BacPAK6
WP5212
(B) T1D
Uninfected
BacPAK6
WP5212
Uninfected
BacPAK6
WP5212
(C) 2° Ab only
Uninfected
BacPAK6
WP5212
(D) CD
Uninfected
BacPAK6
WP5212
(E) T1D/CD
Uninfected
BacPAK6
WP5212

Figure 26. Patients with type 1 diabetes have increased antibody reactivity to WP5212 compared with healthy control subjects. 1D SDS-PAGE and Western blots of WP5212-baculovirus infected insect cell lysate were probed with serum antibodies from control subjects (n = 14) or patients with type 1 diabetes (n = 22). Densitometric analysis of the Western blots was performed to determine antibody reactivity to WP5212. Subjects with antibody reactivity values greater than the mean + 2 SD (2771.9 mean intensity/pixel; red line) of the control group were designated WP5212 antibody positive. Individuals who tested positive for tissue transglutaminase autoantibodies are indicated in red. ANOVA/LSD, * indicates significant difference with control subjects, $p < 0.05$.

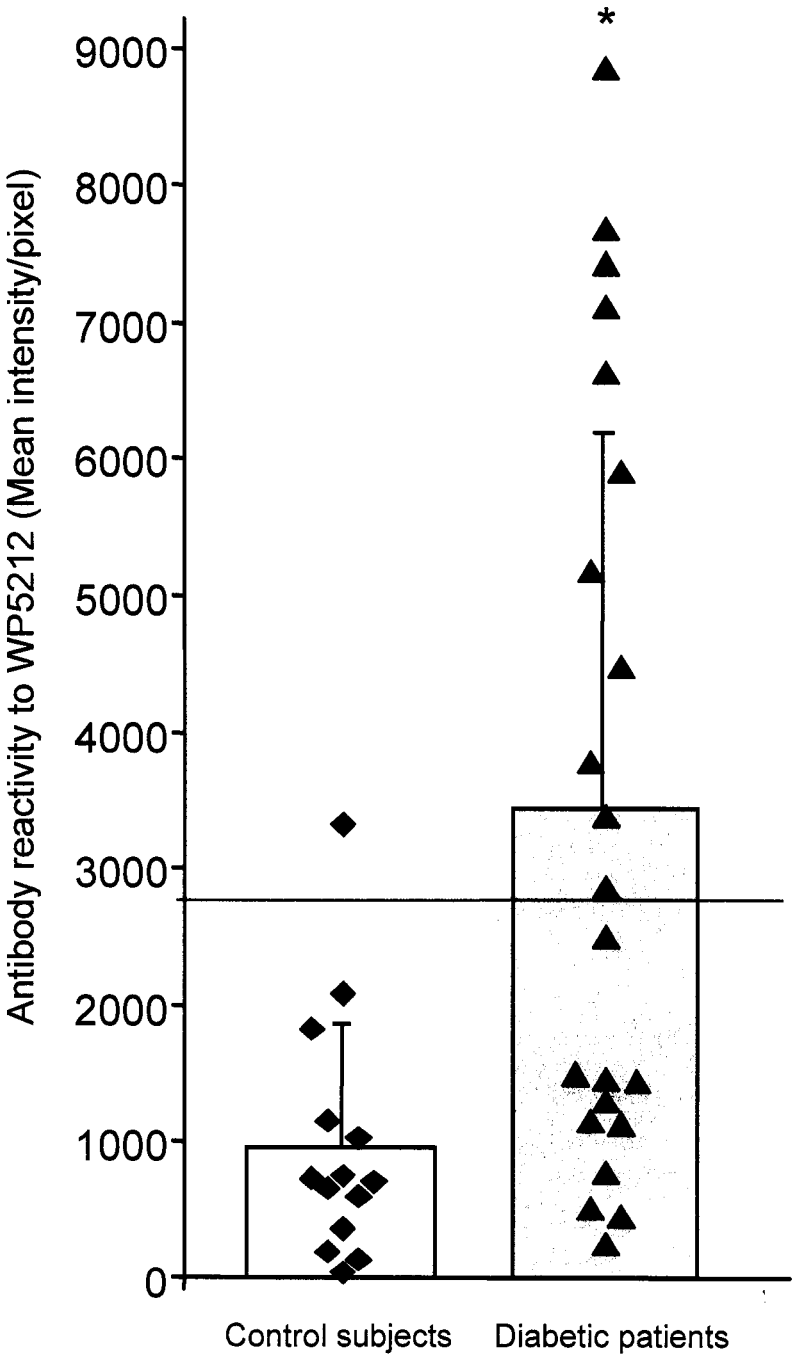
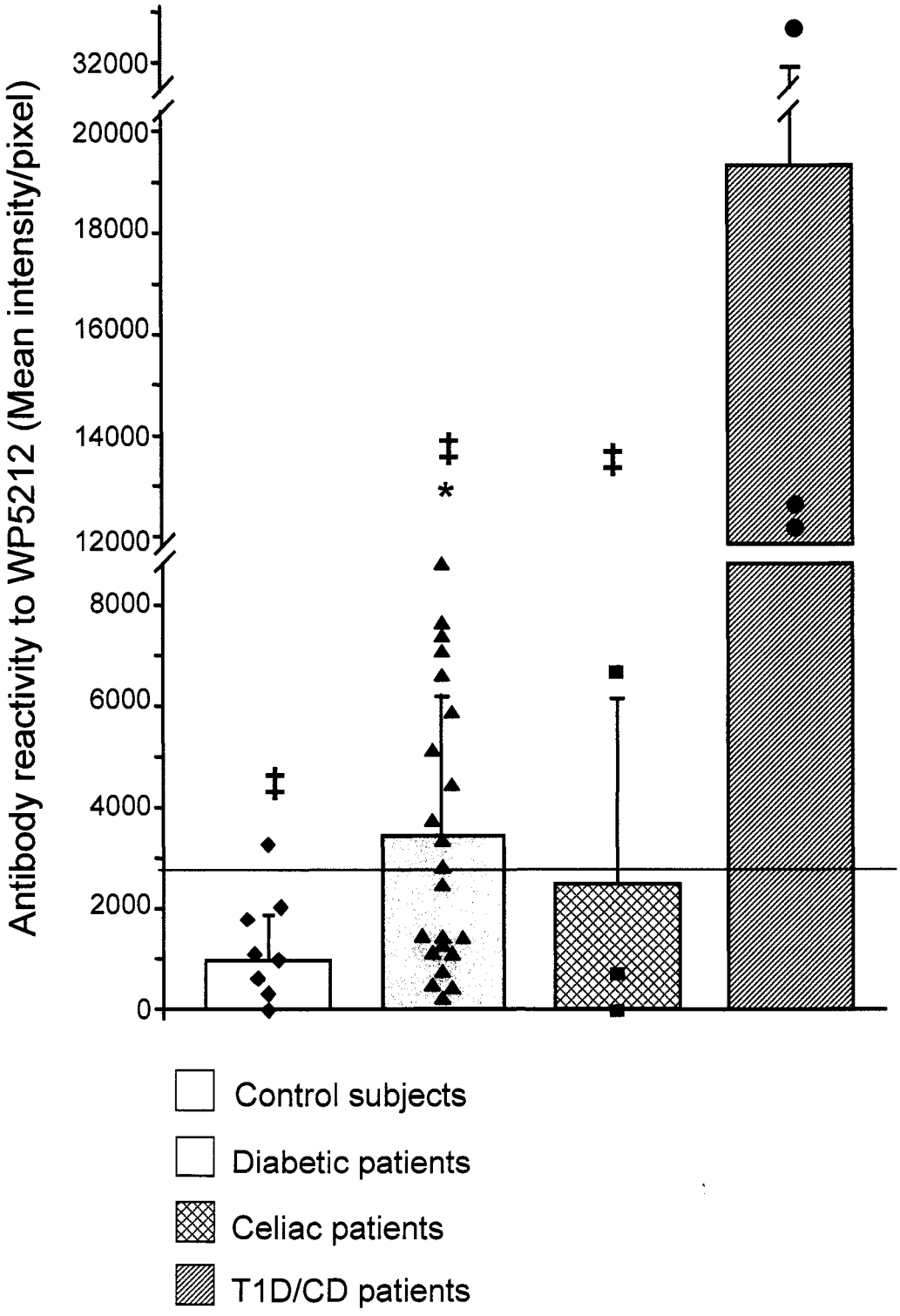

Antibody reactivity to WP5212 (Mean intensity/pixel)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
*
Control subjects
Diabetic patients

Figure 27. Patients with both type 1 diabetes and celiac disease have increased antibodies to WP5212 compared with patients with diabetes alone. 1D SDS-PAGE and Western blots of WP5212-baculovirus infected insect cell lysate were probed with serum antibodies from control subjects (n = 14), patients with type 1 diabetes (n = 22) or celiac disease (n = 3), or both diseases (n = 3). Densitometric analysis of the Western blots was performed to determine antibody reactivity to WP5212. Subjects with antibody reactivity values greater than the mean + 2SD (2771.9 mean intensity/pixel; red line) of the control group were designated WP5212 antibody positive. Individuals who tested positive for tissue transglutaminase autoantibodies are indicated in red; those who tested weakly positive are indicated in blue. ANOVA/LSD, * indicates significant difference vs control subjects, $p < 0.01$; ‡ indicates significant difference vs T1D/CD patients, $p \leq 10^{-5}$.


Antibody reactivity to WP5212 (Mean intensity/pixel)
32000
20000
18000
16000
14000
12000
8000
6000
4000
2000
0
Control subjects
Diabetic patients
Celiac patients
T1D/CD patients

Subjects were designated WP5212 antibody positive when antibody reactivity was greater than the mean intensity plus 2 SD for WP5212 screened with healthy control serum (mean intensity/pixel + 2SD = 2771.9, indicated by the red line on Figures 26 and 27). Diabetic patients (50%) and patients with both diabetes and celiac disease (100%) developed positive antibody titers to WP5212 more frequently than healthy control subjects (7.1%; p=0.008 and p=0.006, respectively; Table 8). Celiac disease patients (33.3%) did not develop WP5212 antibodies more frequently than control subjects.

Production of WP5212 antibodies is not necessarily associated with celiac disease characteristics. Only one of 11 T1D patients with positive WP5212 antibody levels tested positive for autoantibodies to the CD autoantigen, tTG (value 27; Figure 25 and 26). The level of antibody reactivity was median relative to the group of WP5212 antibody positive T1D patients. This T1D patient had the T1D and CD associated risk haplotype HLA-DR3/4, DQ2/* (Table 7). Two of the three T1D/CD patients were tTG autoantibody positive, both of which were deemed positive for WP5212 antibody reactivity. The one control subject that was WP5212 antibody positive was not tTG autoantibody positive.

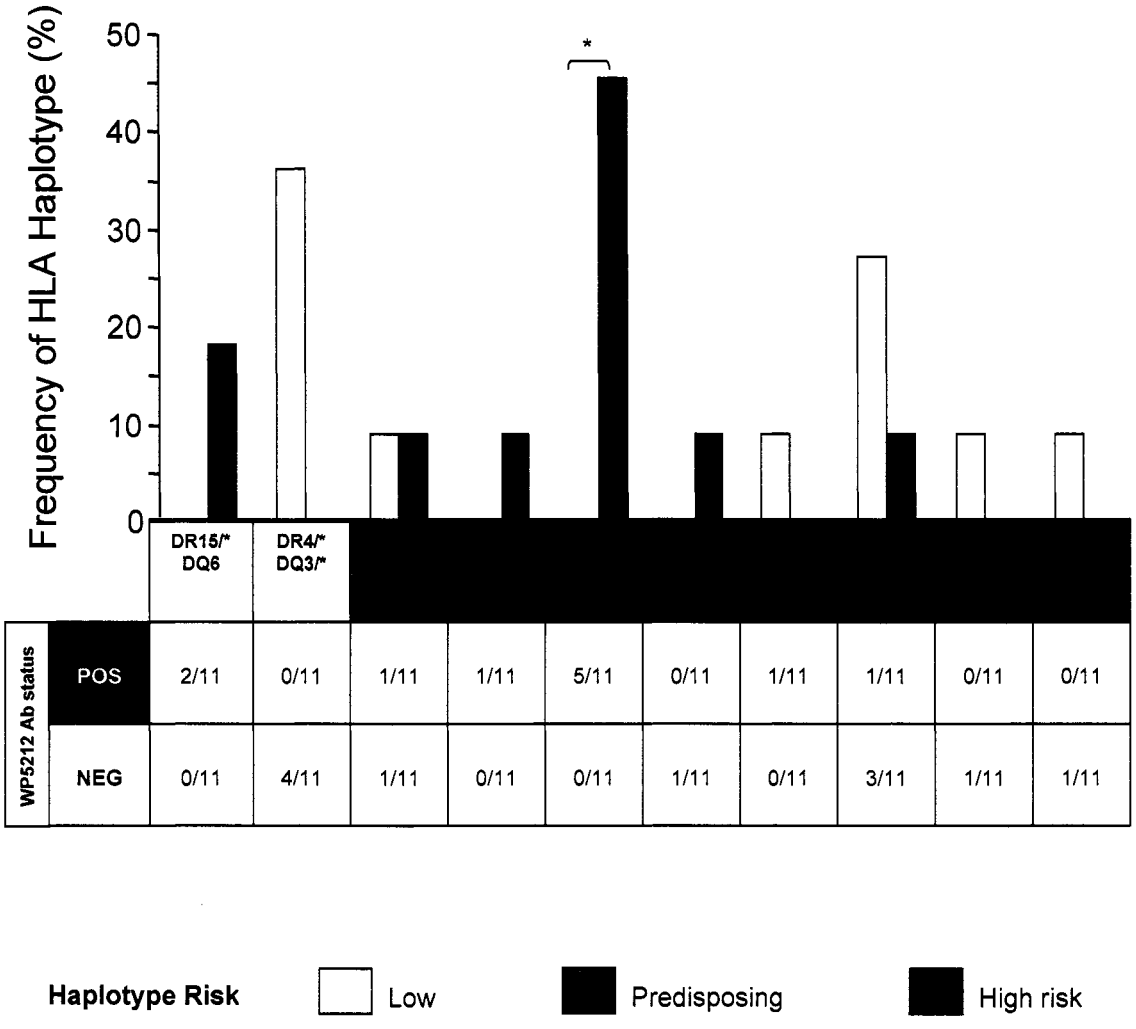
Autoimmune diabetes and celiac disease share a number of HLA-associated risk genes. HLA-DQ2 and DQ8 are both associated with T1D and CD but at different frequencies: 90-95% of CD patients are HLA-DQ2 and 5-10% are HLA-DQ8 (179, 180) while up to 50% of diabetic patients are HLA-DQ8/DQ2 heterozygotes (181). Therefore, it was of interest to determine whether the production of WP5212 antibodies in T1D patients was related with a celiac disease-associated genetic background (Figure 28). Of the 11 WP5212 antibody T1D patients, four did not have a haplotype associated with CD

Table 8. Frequency of WP5212 antibody positive patients

Subject type	Frequency	% Positive	*p* value vs. control[a]
Control	1/14	7.1	--
T1D	1122	50.0	0.008
CD	1/3	33.3	0.3
T1D/CD	3/3	100.0	0.006

[a]Fisher's exact two-tailed test

Figure 28. HLA haplotype and its association with the development of WP5212 antibodies. The HLA haplotypes of type 1 diabetes patients that were deemed positive (filled bars) and negative (open bars) for WP5212 antibody development were compared. The haplotypes are categorized according to diabetes risk: low (yellow), predisposing (orange), high risk (red). Fisher's exact (two-tailed) test, * indicates a significant difference vs. negative patients, $p < 0.05$


Frequency of HLA Haplotype (%)
50
40
30
20
10
0
*
DR15/* DQ6
DR4/* DQ3/*
WP5212 Ab status
POS
NEG
2/11
0/11
1/11
1/11
5/11
0/11
1/11
1/11
0/11
0/11
0/11
4/11
1/11
0/11
0/11
1/11
0/11
3/11
1/11
1/11
Haplotype Risk
Low
Predisposing
High risk

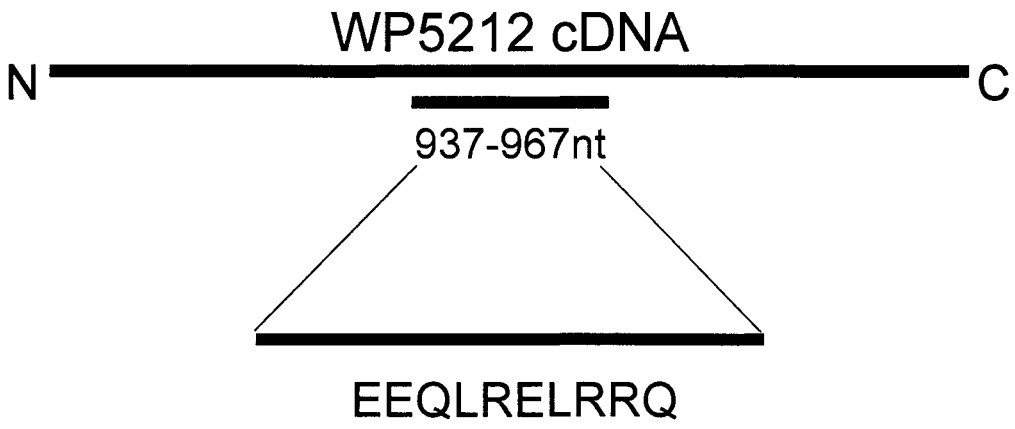
(neither HLA-DQ2 nor DQ8). Six WP5212 antibody positive patients were HLA-DQ2 heterozygous and one was HLA-DQ8 heterozygous, both haplotypes associated with celiac disease. Although 64% of the T1D patients that developed antibodies to WP5212 had haplotypes associated with celiac disease, 36% did not. Of the patients deemed negative for WP5212 antibodies, five did not have haplotypes associated with CD, but four were HLA-DQ2 homo- or heterozygotes and one was an HLA-DQ8 heterozygote.

Study 6: Epitope mapping of the diabetes-related wheat protein WP5212

Identification of a 10 amino acid WP5212 B cell epitope that shares homology with the intermediate filament proteins desmin and vimentin. To determine the antigenic epitopes of WP5212, a library of random, overlapping inserts expressed by transformed cells was screened by colony immunoscreening using serum from the case study patient with both diabetes and celiac disease with high titer WP5212 antibodies. Bacterial colonies expressing antigenic peptides were selected and plasmid inserts were sequenced by using primers complementary to flanking regions of the cloning site in the pSCREEN T-vector. The amino acid sequences from expressed WP5212 peptides were deduced using Clone Manager Professional Suite software. One antigenic clone contained a WP5212 fragment spanning nucleotides 937-967 of the original WP5212 clone (Figure 29, *panel A*). The peptide was expressed in the correct reading frame with the T7 gene 10 fusion protein. It translated to a 10 amino acid sequence (EEQLRELRRQ; Figure 29, *panel A*), which shared 100% (10/10) identity with the expected translated protein sequence of WP5212 (amino acids 309-318). The peptide shared 90% (9/10) identity and

Figure 29. Antigenic epitope of WP5212 and the homologous sequences from the intermediate filament proteins desmin and vimentin, and the glial fibrillary acidic protein. A WP5212 B cell epitope library was constructed and immunoscreened with serum antibodies from a highly wheat sensitive patient with type 1 diabetes and celiac disease. *Panel A*, the identified antigenic epitope corresponds to nucleotides 937 to 967 of the WP5212 open reading frame and translates to a 10 amino acid peptide. *Panel B*, the WP5212 antigenic epitope shares sequence homology with the intermediate filament proteins desmin and vimentin. + indicates a conservative amino acid change.

(A)



(B)

EE+LRELRRQ
Desmin

EE++RELRRQ
Vimentin

EE++R+LR - - - - RQ
GFAP

100% (10/10) positives with the protein sequence of the wheat storage globulin Glb1; there is a conservative change from Q to E at position 10.

To determine whether the antigenic epitope shared homology with self proteins, which could provide a link between WP5212 and the target tissue, a BLAST search of the GenBank protein database was performed (Table 9). Interestingly, the peptide shared 90% (9/10) identity and 100% (10/10) positives with the amino acid sequence for desmin from human, cow, chicken and pig (Figure 29, *panel B;* Table 9). There is a conservative amino acid change from Q to E at position three. It also shared 80% (8/10) identity and 100% (10/10) positives with the protein sequence for desmin from mouse, rat, hamster, teleost fish, sturgeon, frog (two species) and rainbow trout (Table 9). There are two conservative amino acid changes from Q to E at position three and L to M at position four. The epitope also shared 80% (8/10) identity and 100% (10/10) positives with the protein sequence for vimentin (Figure 29, *panel B*; Table 9). There are two conservative amino acid changes from Q to E at position three and L to M at position four. The homology is between WP5212 and vimentin from human, mouse, rat, hamster, frog, cow and viper. The epitope shared 80% (8/10) identity and 100% (10/10) positives with the human cutaneous T-cell lymphoma (CTCL) tumor antigen HD-CL-06. There are two conservative amino acid changes from Q to E and L to M at positions three and four, respectively. The epitope also shared 80% (8/10) identity and 100% (10/10) positives with the zebrafish glial fibrillary acidic protein (GFAP). There are two conservative amino acid changes from E to Q at position one and from Q to E at position three. Human (Figure 29, *panel B*), rat and mouse GFAP also shared homology with the B cell epitope.

Table 9. Proteins with sequence homologies to the WP5212 B cell epitope

Homologous protein [a]	Amino acid homology	Expect value
gi|170696|gb|AAA34269.1| storage protein (wheat)	Query: 1 EEQLRELRRQ 10 EEQLRELRR+ Sbjct: 357 EEQLRELRRE 366	0.58
gi|71541|pir||DMHU desmin - human	Query: 1 EEQLRELRRQ 10 EE+LRELRRQ Sbjct: 155 EEELRELRRQ 164	0.58
gi|2959454|dbj|BAA25134.1| desmin [*Sus scrofa*] - pig	Query: 1 EEQLRELRRQ 10 EE+LRELRRQ Sbjct: 148 EEELRELRRQ 157	
gi|2959452|dbj|BAA25133.1| desmin [*Bos taurus*] - cow	Query: 1 EEQLRELRRQ 10 EE+LRELRRQ Sbjct: 147 EEELRELRRQ 156	
gi|2959450|dbj|BAA25132.1| desmin [*Gallus gallus*] - chicken	Query: 1 EEQLRELRRQ 10 EE+LRELRRQ Sbjct: 139 EEELRELRRQ 148	
gi|22902088|gb|AAC60291.2| fKIAA0552 [*Takifugu rubripes*] teleost fish	Query: 1 EEQLRELRRQ 10 E QLRELRRQ Sbjct: 511 EAQLRELRRQ 520	1.4
gi|31982755|ref|NP_035831.2| vimentin [*Mus musculus*] - mouse	Query: 1 EEQLRELRRQ 10 EE ++RELRRQ Sbjct: 151 EEEMRELRRQ 160	3.4
gi|340219|gb|AAA61279.1| vimentin Homo sapiens - *human*	Query: 1 EEQLRELRRQ 10 EE ++RELRRQ Sbjct: 151 EEEMRELRRQ 160	
gi|207656|gb|AAA42339.1| vimentin *Rattus norvegicus* (Norway rat)	Query: 1 EEQLRELRRQ 10 EE ++RELRRQ Sbjct: 66 EEEMRELRRQ 75	
gi|38303787|gb|AAH45052.2| Vim1 protein [*Xenopus laevis*] - frog	Query: 1 EEQLRELRRQ 10 EE ++RELRRQ Sbjct: 144 EEEMRELRRQ 153	
gi|860908|emb|CAA60679.1| vimentin [*Cricetulus griseus*] chinese hamster	Query: 1 EEQLRELRRQ 10 EE ++RELRRQ Sbjct: 65 EEEMRELRRQ 74	
gi|27806785|ref|NP_776394.1| vimentin [*Bos taurus*]	Query: 1 EEQLRELRRQ 10 EE ++RELRRQ Sbjct: 151 EEEMRELRRQ 160	
gi|17386168|gb|AAL38630.1| vimentin [*Daboia russellii*] Russell's viper	Query: 1 EEQLRELRRQ 10 EE ++RELRRQ Sbjct: 146 EEEMRELRRQ 155	

gi|33186832|tpe|CAE00502.1| TPA: desmin [*Takifugu rubripes*] teleost fish gi|19352179|dbj|BAB85979.1| desmin [*Mesocricetus auratus*] golden hamster gi|38197676|gb|AAH61872.1| Desmin [*Rattus norvegicus*] - rat gi|33563250|ref|NP_034173.1| desmin [*Mus musculus*] gi|32452109|emb|CAD38128.1| desmin [*Acipenser baerii*] Siberian sturgeon gi|64653|emb|CAA34740.1| desmin [*Xenopus laevis*] gi|17221340|emb|CAC83053.1| desmin [*Oncorhynchus mykiss*] rainbow trout gi|45360995|ref|NP_989134.1| desmin [*Xenopus tropicalis*]	Query: 1 EEQLRELRRQ 10 EE ++RELRRQ Sbjct: 131 EEEMRELRRQ 140 Query: 1 EEQLRELRRQ 10 EE ++RELRRQ Sbjct: 155 EEEMRELRRQ 164 Query: 1 EEQLRELRRQ 10 EE ++RELRRQ Sbjct: 155 EEEMRELRRQ 164 Query: 1 EEQLRELRRQ 10 EE ++RELRRQ Sbjct: 155 EEEMRELRRQ 164 Query: 1 EEQLRELRRQ 10 EE ++RELRRQ Sbjct: 145 EEEMRELRRQ 154 Query: 1 EEQLRELRRQ 10 EE ++RELRRQ Sbjct: 144 EEEMRELRRQ 153 Query: 1 EEQLRELRRQ 10 EE ++RELRRQ Sbjct: 141 EEEMRELRRQ 150 Query: 1 EEQLRELRRQ 10 EE ++RELRRQ Sbjct: 144 EEEMRELRRQ 153	3.4
gi|47226295|emb|CAG09263.1| unnamed protein product [*Tetraodon nigroviridis*] fish	Query: 1 EEQLRELRRQ 10 EE ++RELRRQ Sbjct: 363 EEEMRELRRQ 372	3.4
gi|22854562|gb|AAN09720.1| CTCL tumor antigen HD-CL-06 [*Homo sapiens*]	Query: 1 EEQLRELRRQ 10 EE ++RELRRQ Sbjct: 151 EEEMRELRRQ 160	3.4
gi|20977259|gb|AAM33344.1| glial fibrillary acidic protein [*Danio rerio*] - zebrafish	Query: 1 EEQLRELRRQ 10 +E+LRELRRQ Sbjct: 45 QEELRELRRQ 54	4.6
gi|4503979|ref|NP_002046.1| glial fibrillary acidic protein [*H. sapiens*]	Query: 1 EEQLRELR - - - - RQ 10 EE++RELR RQ Sbjct: 205 EEEVRELREQLARQ 218	7.8×10^3
gi|8393431|ref|NP_058705.1| glial fibrillary acidic protein [*R. norvegicus*]	Query: 3 QLRELR 8 +LRELR Sbjct: 117 ELRELR 122	7.8×10^3
gi|26080422|ref|NP_034407.1| glial fibrillary acidic protein [*M. musculus*]	Query: 1 EEQLRELR 8 EE++R+ LR Sbjct: 190 EEEVRDLR 197	5.8×10^3

+ indicates a conserved amino acid change, -- indicates a in the amino acid sequence, a space indicates a non-conserved amino acid change

[a] Homology achieved using the sequence homology matrix PAM-30, recommended for comparing sequences less than 35 amino acids in length.

[b] Homology achieved by a directed pairwise BLAST search.

WP5212 shares structural homology with Jack bean canavalin and soybean conglycinin. To determine the three-dimensional structure of WP5212 and the location of the B cell epitope within WP5212, the translated amino acid sequence was submitted to SWISS-MODEL (167-169). The SWISS-MODEL program superimposes the sequence of interest onto related, solved three-dimensional structures from the Research Collaboratory for Structural Bioinformatics Protein Databank (PDB). The amino acid sequence for WP5212 was aligned with template three-dimensional structures of canavalin from Jack bean (*Canavalia ensiformis*; PDB identification 2CAV and 2CAU) and β-conglycinin from soybean (*Glycine max*; PDB identification 1IPJ and 1IPK). The expected three-dimensional structure of WP5212 can be seen threaded through the known structure of one of the homotrimeric subunits of the soybean β-conglycinin (Figure 30). Based on the predicted structure of WP5212 from both template proteins, WP5212 forms a β-barrel structure interspersed with α-helices and random coils (Figure 31, *panel A* and *B*). The antigenic epitope forms an α-helix on the external hydrophilic surface of the protein (Figure 32).

Antibody cross-reactivity between WP5212 and vimentin. Due to the amino acid sequence homology between WP5212 and the intermediate filament proteins desmin and vimentin, it was of interest to determine whether there existed antibody cross-reactivity between the proteins. 1D SDS-PAGE and Western blots of insect cells expressing WP5212 were performed. The blots were probed with WP5212 antibody positive serum from the case study subject with high WP5212 antibody titers. The serum diluted 1:2000 was preabsorbed with 5 or 50 μg of bovine desmin or recombinant human vimentin, or with 50 μg of β-lactoglobulin as a negative control. Subsequent to Western blotting, the

Figure 30. Predicted three-dimensional structure of WP5212 superimposed on the soybean beta-conglycinin homotrimer. The three-dimensional structure of WP5212 was predicted using SWISS-Model based on similarities with the known three-dimensional structures of soybean conglycinin and Jack bean canavalin. WP5212 is shown in red threaded through one subunit of the soybean conglycinin trimer shown in white. The three-dimensional structure was visualized with DeepView – SwissPDB Viewer software.

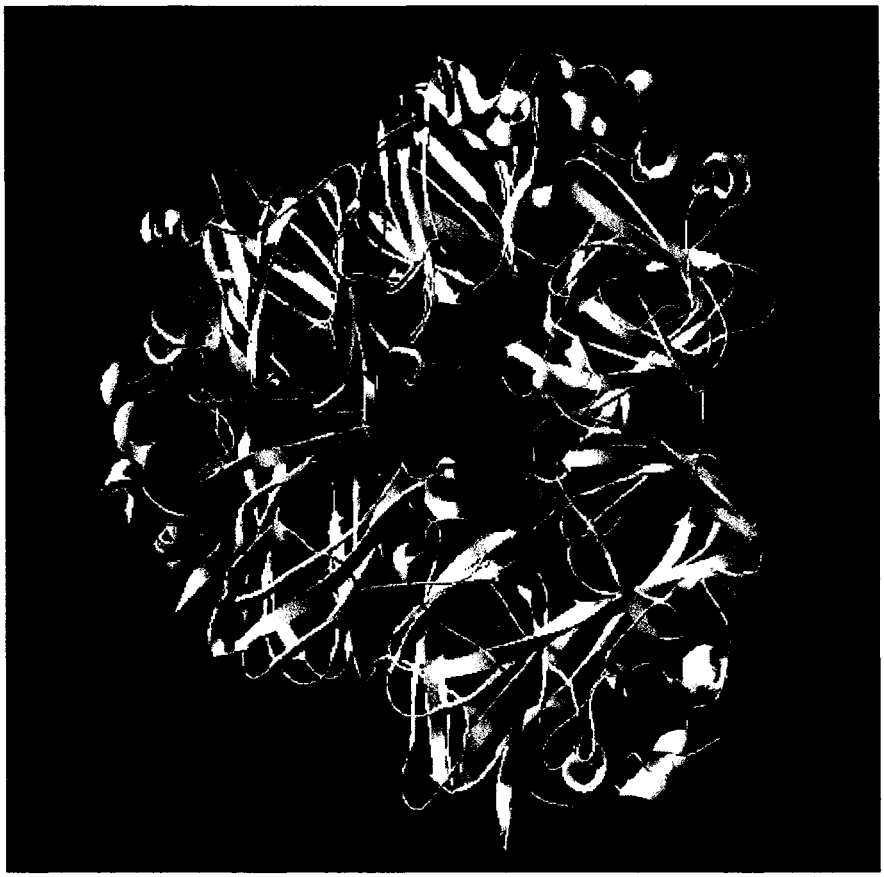


Figure 31. Predicted three-dimensional structure of WP5212. *Panel A*, beta sheets and alpha helices of WP5212 are indicated in yellow and red, respectively. Random coils are shown in blue. *Panel B*, view down the beta barrel formed by the beta sheets of WP5212. The three-dimensional structure was visualized with DeepView – SwissPDB Viewer software.

(A)

(B)

Figure 32. Three-dimensional structure and location of the B cell antibody epitope of WP5212. The antigenic epitope of WP5212 forms an alpha helix shown in green. The three-dimensional structure was visualized with DeepView – SwissPDB Viewer software.

nitrocellulose membranes were reversibly stained with Ponceau S red (nonspecific protein dye) to determine equal protein loading and transfer. There was no decrease in antibody reactivity to WP5212 relative to unabsorbed serum in samples preabsorbed with either 5 or 50 μg of desmin (Figure 33, *panel B* and *A*). However, serum samples preabsorbed with vimentin demonstrated decreased antibody binding to WP5212 as vimentin concentration increased (Figure 34, *panel B* and *C*). There was no difference in WP5212 antibody binding between unabsorbed and β-lactoglobulin preabsorbed serum samples (Figure 34, *panel B* and *C*).

HLA class II epitope predictions for WP5212. HLA class II epitopes for WP5212 were predicted using the SYFPEITHI peptide prediction program (www.syfpeithi.de; (170)). T cell epitopes are predicted based on the likelihood of their being naturally processed and presented in the context of MHC class II molecules. The SYFPEITHI scoring system evaluates every amino acid within a given peptide (in the case of MHC class II, peptides are 15mers). Each amino acid is given an arbitrary value ranging from 1 to 15 for slightly preferred to optimal anchor residues. Negative values are given to amino acids that are likely to have a deleterious effect on peptide binding. The values have been determined based on the frequency of the amino acid in natural ligands, T cell epitopes or binding peptides. The predicted MHC class II motifs are less reliable than those for MHC class I motifs due to higher variability in pocket binding. The reliability is approximately 50%.

WP5212 T cell epitopes were predicted for HLA-DRB1*0301 and HLA-DRB1*0401 haplotypes, as these haplotypes are associated with high risk of diabetes

Figure 33. Preabsorption of WP5212 antibody-positive serum with desmin. 1D SDS-PAGE of WP5212-baculovirus infected insect cell lysate was performed. *Panel A*, Ponceau S red staining of the Western blot was used to determine equal loading of protein (10 μg/lane). *Panel B*, WP5212 antibody positive serum from a patient with both type 1 diabetes and celiac disease was preabsorbed with increasing amounts (0, 5 or 50 μg) of recombinant bovine desmin to determine antibody cross-reactivity between WP5212 and desmin. *Panel C*, densitometric analysis of the Western blot was used to determine absorption of WP5212 antibodies by desmin. β-lactoglobulin (50 μg) was used as a negative control. Data represents the mean of 3 separate experiments.

(A)

(B)

(C)

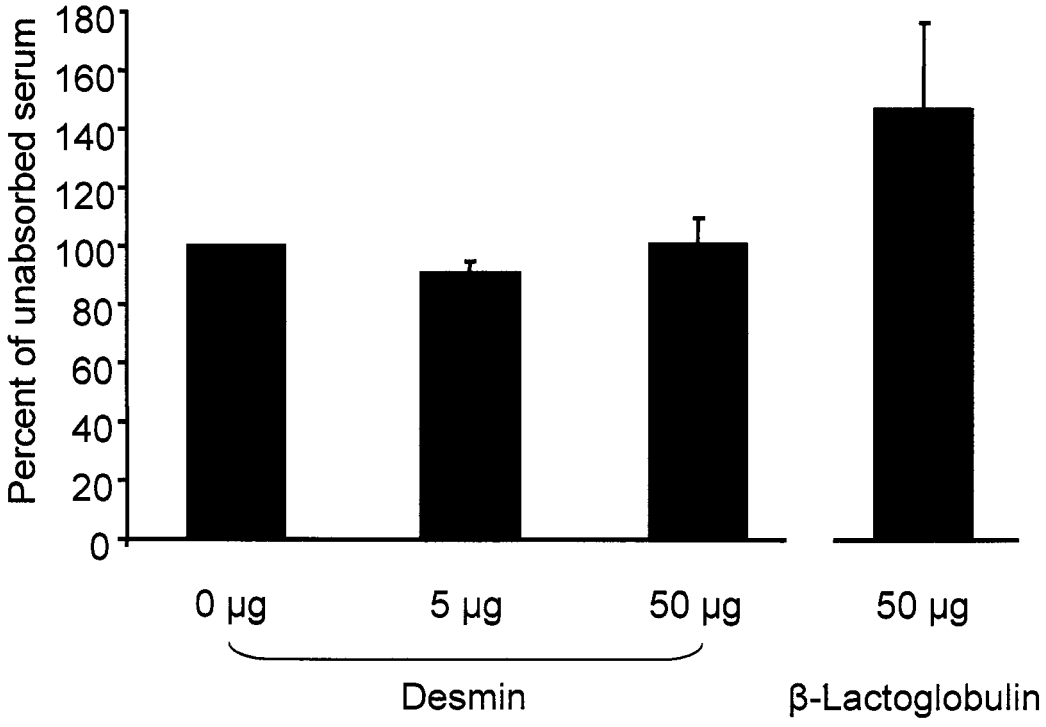
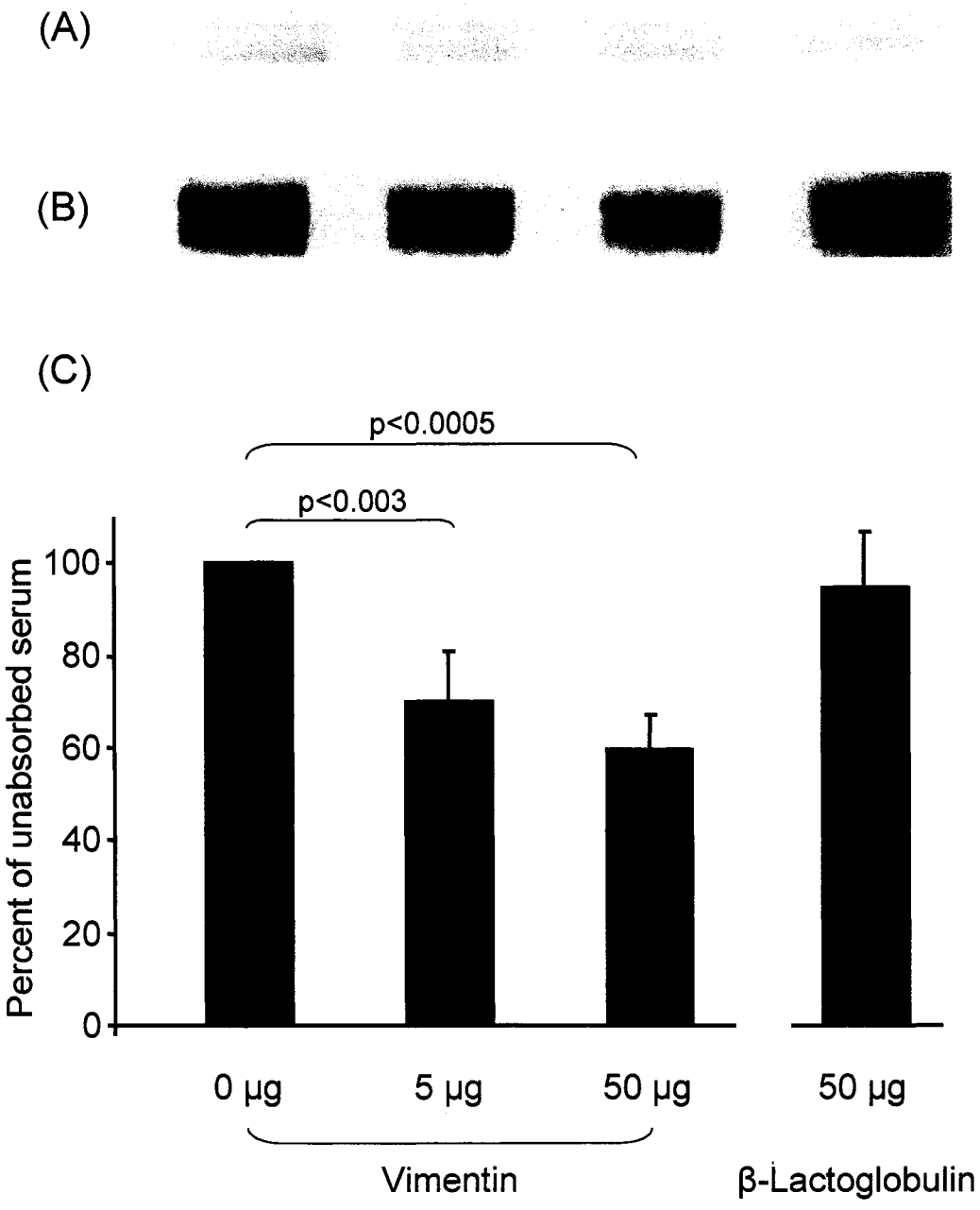

Percent of unabsorbed serum
180
160
140
120
100
80
60
40
20
0
0 µg
5 µg
50 µg
50 µg
Desmin
β-Lactoglobulin

Figure 34. Preabsorption of WP5212 antibody-positive serum with vimentin. 1D SDS-PAGE of WP5212-baculovirus infected insect cell lysate was performed. *Panel A*, Ponceau S red staining of the Western blot was used to determine equal loading of protein (10 μg/lane). *Panel B*, WP5212 antibody positive serum from a patient with type 1 diabetes and celiac disease was preabsorbed with increasing amounts (0, 5 or 50 μg) of recombinant human vimentin to determine antibody cross-reactivity between WP5212 and vimentin. *Panel C*, densitometric analysis of the Western blot was used to determine absorption of WP5212 antibodies by vimentin. β-lactoglobulin (50 μg) was used as a negative control. Data represents the mean of 3 separate experiments. ANOVA/LSD


(A)
(B)
(C)
p<0.0005
p<0.003
Percent of unabsorbed serum
100
80
60
40
20
0
0 µg
5 µg
50 µg
50 µg
Vimentin
β-Lactoglobulin

development. In order to establish a range of acceptable scores for the predicted epitopes, it was essential to define scores for known ligands of the MHC molecules. The highest SYFPEITHI scores for these known ligands are shown as a guide for SYFPEITHI scores determined for the unknown WP5212.

Ligands that are known to bind HLA-DRB1*0301 include *Mycobacterium tuberculosis* antigen 85B (182) and the Lol pollen antigen (183). High and low (high/low) SYFPEITHI scores for these peptides are 12/1 and 20/1, respectively. Therefore, any WP5212 peptides with scores of 12 or higher were considered to be potential T cell epitopes. The highest score achieved for a 15mer peptide from WP5212 was 34. 116 WP5212 peptides had scores equal to or higher than 12, of which 20 peptides had scores equal to or higher than 20 (Table 10), which was the highest score achieved for a known HLA-DRB1*0301 ligand. One of the latter peptides encompassed the entire length of the B cell epitope (score 20) and two other peptides encompassed part of the B cell epitope, including 7 of the N-terminal amino acids (score 26) and 8 of the N-terminal amino acids (score 20; Table 10). The majority of peptides encompassing all or part of the B cell epitope had scores ranging from 1 to 20 (Table 10 and data not shown).

Known ligands for the HLA-DRB1*0401 include GAD65 (184), chlamydial histone-like protein hc-1 (185) and gp100, a tumor cell antigen (186). High and low (high/low) SYFPEITHI scores for these peptides are 18/6, 20/7 and 26/6, respectively. Therefore, any WP5212 peptides with scores within this range 18 or higher were considered to be potential T cell epitopes. The highest score achieved for a 15mer peptide from WP5212 was 28 (Table 11). 77 WP5212 peptides had scores equal to or higher than

Table 10. Predicted HLA-DRB1*0301 class II binding peptides from WP5212

HLA type	Start position in WP5212 amino acid sequence	Score
HLA-DRB1*0301	330	34
	411	27
	231	26
	263	26
	301*	26
	123	25
	204	25
	248	25
	142	22
	516	22
	8	21
	9	21
	10	21
	172	20
	185	20
	276	20
	302*	20
	309	20
	333	20
	505	20

*peptide encompasses part of the length of the B cell epitope
bolded - encompasses the entire length of the B cell epitope

Table 11. Predicted HLA-DRB1*0401 class II binding peptides from WP5212

HLA type	Start position in WP5212 amino acid sequence	Score	Start position in WP5212 amino acid sequence	Score
HLA-DRB1*0401	338	28	38	20
	362	28	141	20
	9	26	142	20
	162	26	158	20
	201	26	173	20
	241	26	191	20
	309	26	221	20
	479	26	227	20
	497	26	228	20
	505	26	239	20
	574	26	245	20
	18	22	262	20
	138	22	263	20
	148	22	270	20
	155	22	282	20
	168	22	301*	20
	254	22	370	20
	256	22	374	20
	330	22	396	20
	355	22	398	20
	386	22	471	20
	457	22	478	20
	506	22	490	20
	526	22	493	20
	6	20	514	20
	8	20	517	20
	16	20	533	20
	17	20		

*peptide encompasses part of the length of the B cell epitope
bolded - encompasses the entire length of the B cell epitope

18 (not all indicated), of which 55 peptides had scores equal to or higher than 20 (Table 10). One peptide encompassed the entire length of the B cell epitope (score 26; Table 11). A second peptide encompassed 7 of the N-terminal amino acids of the B cell epitope (score 20; Table 11). The majority of peptides encompassing all or part of the B cell epitope had scores ranging from 1 to 20 (Table 11 and data not shown). Although rare, it is possible that B and T cell epitopes share homology (187). Therefore, WP5212 contains peptides that may not only be involved in a humoral B cell response but also a cellular immune response associated with the development of type 1 diabetes.

DISCUSSION

Type 1 diabetes results from the targeted immune-mediated destruction of the insulin-secreting β cells in the pancreatic islets of Langerhans. The development of autoimmune diabetes involves complex interactions among susceptibility and resistance genes, environmental triggers and enhancers, and an underlying vulnerability of the target tissue to damage (Figure 1 and 3). Until recently, the intricacies of diabetes pathogenesis have not been fully appreciated. Over 20 genes with varying penetrance and numerous candidate environmental agents add a high level of complexity to the problem of defining the mechanisms involved in the development of diabetes in humans.

Most candidate environmental factors associated with human T1D have been identified using epidemiological data. A few reports of infectious outbreaks and diabetes and the presence of viral antibodies in T1D patients and their first degree relatives have implicated viruses in disease development. Although several candidate viruses have been proposed, none has been proven to cause diabetes in humans. In fact, a recent study found that germ-free diabetes-prone BB rats still develop diabetes, which leaves diet as the main source of environmental stimuli and promoter of islet autoimmunity (Scott *et al*, unpublished data).

Several lines of evidence suggest that wheat is a major dietary contributor to diabetes pathogenesis. Diets in which wheat is the sole or major amino acid source induce nearly three times as many cases of diabetes in diabetes-prone BB rats and more than twice as many cases in non-obese diabetic mice, in comparison with a protective hydrolyzed casein-based diet (Figure 4 and 15) (49, 116, 122, 127, 128, 130). Similar

studies have shown that gluten-free diets are protective in NOD mice in comparison with gluten-containing diets (131, 132).

Human studies also support a role for cereal proteins in the development of diabetes. Two large independent prospective studies found that early oral exposure to cereal proteins increased the risk of islet autoimmunity in children genetically at-risk for developing diabetes. Children introduced to cereals earlier than 3 months or beyond 7 months of age (147) or more specifically, those introduced to gluten-containing cereals before 3 months of age were at increased risk of islet autoimmunity (148). These studies suggest that exposure to cereals and the timing of exposure can affect diabetes risk. Furthermore, there are data indicating that wheat proteins can be immunostimulatory in both animal models of T1D and diabetic patients (150-152, 154). Despite all of the data that support a role for dietary wheat proteins in diabetes development, the identity of the culpable wheat antigens remains unknown.

We have successfully identified the first candidate diabetes-related dietary wheat protein. Without knowledge of the specific molecular structures involved in triggering or enhancing autoimmune diabetes, the pathogenic mechanisms cannot be elucidated, which in turn impedes the development of targeted preventive and therapeutic measures. The conundrum is that without knowledge of the mechanism by which an antigen is pathogenic, it is difficult to identify specific offensive molecules. Further confounding the situation is the difficulty in linking past exposures to environmental agents with later development of diabetes among a large number of different risk genotypes. These hurdles were overcome by using antibodies from diabetic individuals to sift through the proteome of the wheat endosperm. This unique approach of immunoscreening a wheat cDNA

expression library permitted us to identify the first candidate diabetes-related wheat protein.

Identification of the first candidate diabetes-related dietary wheat protein[1]

The primary goal of the project was to identify unique diabetes-related wheat proteins. To analyze as many potential diabetes-related wheat proteins as possible, over one million recombinant phage from a wheat cDNA expression library were screened with pooled sera from diabetic BB rats. Eight positive clones were isolated and by restriction enzyme analysis and nucleotide sequencing were shown to contain three distinct sets of cDNA inserts (Figure 8). A representative clone from each distinct set, WP12111, WP23112 and WP5212, was used for all further analyses. Upon immunoscreening the clones with antibodies from individual diabetic, asymptomatic and control rats, it was found that antibody reactivity against WP5212 was strongest in diabetic individuals. IgG reactivity against WP5212 was strain-specific and dependent on diabetes risk: highest in overt diabetic, lower in asymptomatic BB rats and lowest in non-diabetes-prone BBc rats (Figure 11, *panel B*). BLAST searches revealed that clone WP5212 shared 100% homology with the wheat EST clone TC103916 (Figure 9; Table 2). It also showed high similarity at the nucleotide and translated amino acid level with the wheat storage globulin, Glb1 (Figure 10; Table 2).

It was important to link antibody development to the identified wheat proteins with damage to the target tissue. Mononuclear cells increasingly infiltrate the β cell containing core of the islets during the progression of autoimmune diabetes, a process

[1] This section reflects the article entitled "A type 1 diabetes-related protein from wheat (*Triticum aestivum*). cDNA clone of a wheat storage globulin, Glb1, linked to islet damage" by MacFarlane *et al* published in the *Journal of Biological Chemistry* volume 278 issue 1 in January 2003 (49).

called insulitis. The extent of damage to the pancreas is reflected by the severity and prevalence of destructive insulitis, loss of islet β-cells and ultimately hyperglycemia. As expected, rats with overt diabetes had fewer islets, higher percent of islets infiltrated with mononuclear cells and higher mean insulitis scores than either asymptomatic BBdp rats or control rats (Table 3). Asymptomatic BBdp rats that did not develop diabetes by 150 days of age had a higher percent of infiltrated islets and higher mean insulitis score in comparison with BBc rats. Thus, a continuum was observed in which inflammation and islet damage increased from control to asymptomatic to overt diabetic rats reflecting an increasing risk for diabetes development.

It was of interest to determine whether strongly reactive antibodies to WP5212 from BBdp rats correlated with the destructive immune process that targets the pancreatic islet β-cells. In patients with type 1 diabetes, the presence of autoantibodies to either GAD or IA-2 has been shown to be closely correlated with *in situ* pancreatic insulitis and/or hyperexpression of MHC class I molecules in islets (42). When sera from individual rats at different risk of developing diabetes were used, IgG reactivity against the WP5212 clone closely correlated with overall pancreas infiltration and damage, as well as inflammation of individual islets (Figure 12, *panels A* and *B*). The other two clones, WP12111 and WP23112, which shared amino acid homology with putative *A. thaliana* proteins, showed no correlation between antibody reactivity and islet inflammation or damage (Figure 12, *panels C-F*). Therefore, these clones were not investigated further. The close correlation between antibody reactivity to WP5212 and islet inflammation in diabetes-prone and diabetic BB rats represents a new and unique association between a previously unidentified wheat antigen and the target tissue.

A diabetes-related wheat storage globulin hidden among wheat gluten proteins[1]

The wheat seed endosperm consists of starch granules embedded in a complex matrix composed mostly of storage proteins. The endosperm proteome is complex due not only to the tremendous number of proteins, as many as 1300 proteins can be identified by two-dimensional electrophoresis, but as well by their large size, physicochemical properties and functions (188). Traditionally, wheat proteins have been classified according to their solubility. The gliadins and glutenins, which are soluble in aqueous alcohol and dilute acid/alkali, respectively, are the major storage proteins making up 80% of the total protein. The water soluble albumins and salt-soluble globulins are minor components making up only about 20% of the total protein (189). However, many proteins fall into more than one solubility classification. These characteristics compound the problems that already exist in the identification of specific diabetes-related proteins.

The wheat protein used to supplement the WP-diet consisted of a mixture of wheat gluten proteins. WG consists of a large macromolecular polypeptide complex, the majority being gliadin and glutenin proteins, which have been concentrated after repeated extraction of wheat flour with water. This process removes starch, and the majority of albumins and globulins. Based on its predicted amino acid sequence, WP5212 is classified as a 7S globulin. Therefore, it was rather surprising to identify it as a major diabetes-related wheat protein in animals fed a WG-based diet. Thinking along traditional lines, WP5212 should be absent or at most a minor constituent in the water-extracted WG diet. However, as mentioned, wheat proteins are difficult to separate into distinct classes based on solubility and globulin proteins have been shown to remain in the wheat gluten

fraction (190). In a complementary study aimed at identifying diabetes-associated WG proteins, a protein sharing homology with both the wheat storage globulin Glb1 and clone WP5212 was identified in extracts of WG by two-dimensional Western blotting and mass spectrometry (49). Pooled antibodies from newly diagnosed, insulin-untreated diabetic patients bound this Glb1/WP5212 homologue while pooled antibodies from age, sex and HLA-DQ-matched control subjects did not (49). The data from this study confirmed the identity of WP5212 as a candidate diabetes-related wheat protein and showed that this protein is present at low levels in WG extracts.

Cereal crops, such as maize, rice, sorghum and wheat, diverged from a common ancestor 50 to 70 million years ago (191). Despite a 35-fold difference in genome size, the gene repertoire and the linear arrangement along the chromosomes of diverse cereals have evolved slowly (192). This is because the evolution of the overall genome size and organization is largely due to the generation and elimination of mobile dispersed repetitive DNA elements (192, 193). Thus, it is not surprising that proteins within and among cereal species share related sequence and structural similarities. Because of these similarities across cereals, it was of interest to determine whether WP5212 homologues exist in other cereals. WP5212 shares significant nucleotide sequence identity with 7S globulins, specifically Glb1-like vicilins in barley, rice, maize, sorghum and sunflower (Table 4). WP5212 also shares sequence homology with proteins in the cereal rye and in two dicotyledonous vegetable plants, soybean and tomato (Table 4). Furthermore, when the three-dimensional structure of WP5212 was predicted using the SWISS-MODEL program, it was found to share structural homology with soybean conglycinin (Figure 28) and Jack bean canavalin. These findings suggest that WP5212 shares both sequence and

structural homology with proteins found in other cereals and vegetable plants. Thus, other plants could also harbor diabetes-promoting proteins.

WP5212 shares homologies with food allergens and self proteins

Immunomodulatory proteins have been proposed to engage the immune system by similar mechanisms using shared structures. Similarities have been identified among plant food allergens allowing for their classification by structural or functional properties (194). An example is the allergenic 2S albumins and cereal seed α-amylase/trypsin protease inhibitors, which are part of the prolamin superfamily (194, 195). Furthermore, there are immunologically relevant structural similarities among wheat proteins as demonstrated by cross reactivity of monoclonal antibodies among conserved epitopes in albumins and globulins, or gliadin and glutenins (196, 197). To determine whether WP5212 shares homologous sequences and structures with known immunomodulatory proteins, general searches and specific pair-wise comparisons were performed. WP5212 shares amino acid sequence homologies with an important immunomodulatory food protein, the peanut allergen, Ara h I. This member of the vicilin seed storage family is a major allergen in more than 90% of peanut-sensitive patients (198). The antibody-binding epitopes of the peanut allergen Ara h 1 have been mapped and WP5212 shares identity with three out of four immunodominant B cell epitopes, and four out of five other commonly recognized epitopes (171). Also, the WP5212 protein shares sequence homology with a known soybean allergen (Table 4). This is notable because globulins are the major protein constituent (90%) of soybean, a protein source that has been reported to promote the development of diabetes in BBdp rats, albeit to a lesser extent than the WP

diet (116, 199). The data suggest that these food proteins could have common immunomodulatory and potentially diabetes-promoting epitopes.

Environmental modification of autoimmunity has been hypothesized to occur by molecular mimicry, resulting in immune cross-reactivity between environmental antigens and self proteins (158, 172). Sequence homologies were observed between WP5212 and tight junction proteins (Table 4), which make up part of a protein complex that controls the permeability of the intestinal epithelium. This is interesting because abnormally increased gut permeability to mannitol, a marker of paracellular transport, has been reported in prediabetic BB rats (200) and newly diagnosed patients with type 1 diabetes (201). Cross reactivity between WP5212 and tight junction proteins could lead to damage of the gut mucosa making it more permeable to dietary antigens, causing normal oral tolerance mechanisms to be overwhelmed, leading to increased immune reactivity to dietary wheat proteins that in a subset of susceptible individuals promotes the development of diabetes.

The WP5212 B cell epitope shared amino acid homology with the glial fibrillary acidic protein (GFAP) in zebrafish (Table 9). GFAP was recently identified as a specific immunological target of the destructive autoimmune process in NOD mice and patients with T1D (202), so it was of interest to determine whether homologies between mammalian GFAP and the WP5212 B cell epitope also existed; indeed, it does (Table 9). The islets of Langerhans are enveloped by Schwann cells, which express GFAP (203), and which separate the exocrine and endocrine tissues (202). It was found that NOD mice and T1D patients develop B and T cell responses to GFAP and that targeted destruction of the pancreatic Schwann cells is an important part of disease progression (202).

Considering the data at hand, immune cross-reactivity between WP5212 and GFAP could induce this early step in the development of diabetes.

Prediabetic NOD mice develop diabetes-associated WP5212 antibodies

Cereal-based diets influence diabetes outcome in a second rodent model of spontaneous autoimmune diabetes, the NOD mouse. NOD mice fed a mainly wheat-containing diet develop diabetes with an incidence more than twice that of animals fed a protective hydrolyzed casein diet (Figure 15). To support the hypothesis that the wheat storage globulin WP5212 is associated with diabetes, as was observed in BBdp rats, WP5212, as well as WP12111, WP23112 and WPCON, were immunoscreened with serum antibodies from prediabetic NOD mice. Prediabetic female NOD mice developed antibodies to WP5212 with increased reactivity in comparison to asymptomatic NOD mice and control NOR mice (Figure 16, *panel A* and *B*). Development of WP5212 antibodies was specific to the diabetes-susceptible NOD mouse strain (Figure 16, *panel C*). The data from this second species mirror those observed for BBdp rats and are therefore a strong indicator that WP5212 is abnormally antigenic and could have a role in the development of spontaneous autoimmune diabetes.

The trend of increasing antibody reactivity with increasing diabetes risk was not as clear in NOD mice as that observed in overt diabetic BBdp rats. The difference between diabetic and asymptomatic mice was not significant when the Scheffé's post-hoc statistical test was used. In asymptomatic BBdp rats, the level of insulitis is significantly less than seen in overt diabetic BB rats (Table 3), and it was hypothesized that increased antibody reactivity to WP5212 reflected the extent of islet autoimmunity. In contrast to

BBdp rats, 100% of NOD mice develop insulitis by 20 weeks but not all of the animals progress to overt diabetes (62). It was also observed that there was no difference in insulitis rating between end of study diabetic and asymptomatic NOD mice (data not shown). Therefore, WP5212 antibody reactivity may still reflect damage to the target tissue and islet autoimmunity, but does not differentiate the degree of inflammation between diabetic and asymptomatic animals.

Increased WP5212 antibody reactivity is associated with diabetes in humans

Wheat has been implicated in the development of diabetes in humans. Two large independent prospective studies of children genetically at-risk of developing diabetes found that early oral exposure to cereal proteins increased the risk of islet autoimmunity (147, 148). Also, wheat proteins can stimulate the immune system in both BBdp rats and NOD mice (128, 150, 151), as well as in T1D patients. Increased T cell stimulation in response to wheat gluten was observed in T1D patients in comparison with control subjects in the absence of CD (152)(Scott *et al*, unpublished data). Furthermore, rectal and jejunal lamina propria and epithelium $CD3^+$ and $\gamma\delta^+$ lymphocytes respond to gliadin in 20% of diabetic children who display no clinical signs of CD (153, 154). Now that a candidate diabetes-related wheat protein was identified, it was of interest to determine whether antibody production to WP5212 was linked to diabetes development in human T1D patients.

We were introduced to a 28 year old Caucasian woman who had both type 1 diabetes and the wheat gluten sensitive enteropathy, celiac disease. This patient was highly wheat sensitive. Her symptoms included multiple bowel movements per day, oral

ulcerations and severe lip swelling (Figure 19). After diagnosis of CD, the initiation of a standard gluten-free diet gave respite from symptoms for a short period of time, however, her symptoms returned and worsened. It was not until the patient self-initiated a strict specific-carbohydrate, cereal-free diet that symptoms were alleviated.

Because of her extreme sensitivity to cereal proteins in combination with autoimmune diabetes, it was of interest to determine whether she had antibodies to WP5212. Indeed, the patient developed strongly reactive WP5212 antibodies in comparison with healthy control subjects (Figure 21). The specificity of the WP5212 antibodies was high relative to the control wheat protein WPCON (Figure 20). The patient did not have detectable antibodies to the other candidate proteins, WP12111 or WP23112 at higher serum dilutions (Figure 20 and 21). This was the first indication that as in BBdp rats, WP5212 could be linked to diabetes in humans.

To validate further the relationship between the wheat storage globulin WP5212 and diabetes in humans, patients with diabetes and age and sex-matched healthy control subjects were screened for WP5212 antibodies. To better measure antibody titer, a system was developed to standardize the quantity of protein screened. Markus Walz in the lab of Hubert Kolb (German Diabetes Research Institute, Heinrich Heine University of Düsseldorf, Germany) constructed a recombinant WP5212-baculovirus for expression of the protein in insect cells. The identity of WP5212 was confirmed by probing Western blots of WP5212-baculovirus infected Sf21 insect cell lysate with WP5212-specific polyclonal antibodies (Figure 24). 1D SDS-PAGE of equal quantities of WP5212-baculovirus infected insect cell lysate (10 μg/lane) and Western blots were probed with serum antibodies from individual patients with type 1 diabetes, celiac disease, both

diseases or healthy control subjects (Figure 25). The strength of IgG antibody reactivity was measured by densitometric analysis.

Remarkably, half of the diabetic patients showed an increase in mean antibody reactivity to WP5212 in comparison to healthy control subjects (Figure 26). Also, the frequency of WP5212 antibody positivity was more than seven times higher in the diabetic group than in the control group (50 vs. 7%, ANOVA/LSD, $p = 0.008$; Figure 26; Table 8). In addition, it was striking that the three patients with both T1D and CD displayed the highest (individual and combined) mean antibody reactivity to WP5212 in comparison with healthy control subjects, as well as patients with T1D or CD (Figure 27; Table 8). Further studies are required to determine whether strong antibody reactivity to WP5212 is a marker of extreme wheat sensitivity that is attributable to a synergism between two wheat-related (gut) immune-mediated diseases. Another unexplored point is the possibility that WP5212 antibodies could be used as a diagnostic indicator of CD susceptibility or progression in patients with diabetes.

Because celiac disease is the result of an immune reaction to wheat proteins and could contribute to the production of WP5212 antibodies, WP5212 antibodies were evaluated in patients with CD alone. One of three CD patients was WP5212 antibody positive and there was no difference in mean antibody reactivity between the diabetic and CD patients (Figure 27; Table 8). However, the number of CD subjects included in this study was low and their HLA haplotypes were not determined, so a genetic association with WP5212 antibody production could not be determined. The data can be interpreted in a number of ways. Either WP5212 antibodies are not specific for diabetes, or they are diagnostic or prognostic of wheat-related diabetes in a subset of susceptible individuals.

WP5212 antibody reactivity could also be a marker of enhanced gut immune reactions to dietary wheat proteins or gut damage. It will be necessary to study a larger number of CD and T1D/CD patients to clarify the T1D-dependent or independent relationship between WP5212 antibodies and CD development.

A large proportion of patients with autoimmune diabetes, as much as one third, have been shown to develop autoantibodies to the celiac disease associated autoantigen tissue transglutaminase (143, 204). The presence of anti-tTG antibodies could indicate subclinical CD and thus confound the finding of high WP5212 antibodies in T1D patients. Therefore, tTG antibodies were measured in all subjects studied (Table 7). One patient with CD tested weakly positive for tTG antibodies, likely reflecting recent dietary exposure to gluten proteins, and two patients were negative. No control subjects tested positive for tTG antibodies. All three of the T1D/CD patients were positive for tTG antibodies despite following a gluten free diet. The one weakly tTG antibody positive T1D/CD patient was the case study subject who was following a strict specific-carbohydrate, cereal-free diet. The persistence of tTG antibodies in these patients further suggests that they are at the extreme upper end of wheat sensitivity. One T1D patient out of 22 was positive for tTG autoantibodies. This T1D patient had the HLA-DR3/4, DQ2/* haplotype and thus shared genetic risk for both type 1 diabetes and celiac disease. An intestinal biopsy will be required to determine whether this patient has celiac disease. Interestingly, this patient was also positive for WP5212 antibodies, but with median antibody reactivity in comparison to the other T1D patients that had positive WP5212 antibody reactivity. The data suggest that antibodies to WP5212 could be used as a marker of a subset of T1D patients with wheat-related diabetes. Furthermore, the

presence of strong WP5212 antibody reactivity in T1D patients could indicate susceptibility to the future development of CD.

The incidence of CD is as much as 16 fold higher in T1D patients compared with the general population (204). Of patients with both T1D and CD, 90% develop T1D first, suggesting that after CD diagnosis individuals placed on a gluten-free diet are less likely to develop diabetes. Patients with autoimmune diabetes and celiac disease share a number of HLA-associated risk genes. HLA-DQ2 and DQ8 are both associated with T1D and CD but at different frequencies: 90-95% of CD patients are HLA-DQ2 and 5-10% are HLA-DQ8 (179, 180) while up to 50% of diabetic patients are HLA-DQ8/DQ2 heterozygotes (181). The high coincidence of T1D and CD has been attributed to shared genetic risk. Therefore, it was of interest to determine whether the production of WP5212 antibodies in T1D patients was related to a celiac disease-associated genetic background (Figure 28). Of the 11 WP5212 antibody positive T1D patients, four did not have a haplotype associated with CD (neither HLA-DQ2 nor DQ8). Six WP5212 antibody positive patients were HLA-DQ2 heterozygous and one was HLA-DQ8 heterozygous, both haplotypes associated with celiac disease. Thus, 64% of the T1D patients that developed antibodies to WP5212 had haplotypes associated with celiac disease, whereas 36% did not. Of the patients deemed negative for WP5212 antibodies, five did not have haplotypes associated with CD, but four were HLA-DQ2 homo- or heterozygotes and one was an HLA-DQ8 heterozygote. Together, the data suggest that the development of WP5212 antibodies is not necessarily linked to a CD genetic background, since not all WP5212 antibody positive diabetic patients had CD-associated HLA haplotypes. Because of the large number of HLA haplotypes, a larger study must be performed to clarify the relationship

between genetic susceptibility and WP5212 antibody development.

WP5212 antibody development is diet-dependent: Linking the gut with diabetes

In both BBdp rats and NOD mice, WP5212 antibody production was dependent on a mainly wheat, cereal-based diet (Figure 14 and 17, respectively). Therefore, antibodies to WP5212 act as a biomarker of wheat-related diabetes, unlike diabetes that is solely dependent on genetics, as observed in HC-fed diabetic animals. The dependence on diet for antibody production to this wheat protein (and likely other dietary wheat antigens) provides a potential link between diabetes development and the immune system of the gastrointestinal tract. Oral tolerance appears to be impaired in diabetes susceptible individuals, which is supported by the parallel decrease in WP5212 antibody reactivity in diabetes resistant NOR mice and BBc rats. The intestine encounters the most antigens in comparison with all other organs in the body. In order to interpret this massive amount of environmental stimulation, the gastrointestinal tract develops the largest number of immune cells (205). The gut-associated lymphoid tissues (GALT) are normally tolerant, or immunologically hyporesponsive to harmless environmental antigens, such as commensal bacteria and dietary proteins (206). However, the GALT also has the capacity to induce a strong protective immune response against pathogens (205). The inductive and effector sites of the GALT include the Peyer's patches (PPs), which are macroscopic lymphoid aggregates in the submucosa along the length of the intestine, the lamina propria (LP) located within the intestinal villi, and the mesenteric lymph nodes (MLNs) that drain both the PPs and the LP (207, 208). Pathogens gain access to the GALT via specialized antigen handling microfold (M) cells located in the PP epithelial dome (207).

Professional APCs residing in the PPs or MLNs present pathogenic antigens to naïve $CD4^+$ T cells. The activated $CD4^+$ T cells increase the expression of the integrin $\alpha4\beta7$ and chemokine receptor CCR9, which aid in lymphocyte recirculation through the intestinal mucosa (209, 210). Dendritic cells residing in the PPs normally secrete IL-10, a cytokine associated with a Th2 or regulatory T cell-mediated immune responses that are conducive to the development of oral tolerance (211). However, DCs in the PPs can be induced to secrete the inflammatory cytokine IL-12 such that T cells are primed to be gut-homing T_H1 cells that secrete IFNγ resulting in local or systemic inflammation (208). Thus, WP5212 in the diet likely stimulates a T cell response (as indicated by the presence of IgG antibodies) in the gut-associated lymphoid tissues.

The gut-homing ability of T cells activated in the GALT is due to the expression of the integrin $\alpha4\beta7$. This molecule has been implicated in the development of diabetes. When α4 is depleted using monoclonal antibodies, NOD mice are significantly protected from developing spontaneous diabetes and they cannot adoptively transfer disease (212-215). Also, female NOD mice lacking integrin β7 (NOD.Itgb7$^{-/-}$) show a delayed onset of diabetes and an overall decrease of disease incidence (approximately 35% vs. 95% in female NOD mice; personal communication, E. Leiter). The loss of integrin β7 results in decreased lymphocyte populations in the Peyer's patches and to a lesser extent in the mesenteric lymph nodes (personal communication, E. Leiter). It was found that NOD.Itgb7$^{-/-}$ mice in the prediabetic period developed antibodies to WP5212 with significantly decreased reactivity in comparison with prediabetic and asymptomatic NOD mice and they were negative for WP5212 antibodies (Figure 18), as defined by the criteria for prediabetic and asymptomatic NOD mice (Figure 16). These data suggest that

integrin β7 is required for WP5212 antibody production and that activation of an immune response to WP5212 occurs in the GALT.

Gut-associated lymphocytes, as defined by the expression of integrin α4β7, have been implicated in the development of diabetes in the NOD mouse (216, 217). Mucosal addressin cell adhesion molecule-1 (MadCAM-1), the ligand for α4β7, is expressed on vessels in the exocrine pancreas and on endothelial cells in and around the islets during the early stages of insulitis. As lymphocytes accumulate in the islets of NOD mice, MAdCAM-1 expression on islet vessels increases (218, 219). Concomitantly, α4β7 is expressed at high levels on the majority of infiltrating leukocytes in the islets (216, 217). Before 12 weeks of age 50% or more of the infiltrating lymphocytes in the pancreas express integrin β7 (219). Also, late phase insulitis is likely mediated by α4β7, as it permits both lymphocyte rolling and strong adhesion to endothelial cells in the mucosal lymphoid tissues (220, 221). Diabetes development is inhibited through the use of monoclonal antibodies specific for α4 integrin by blocking T cell binding to pancreatic endothelium and pancreatic lymph nodes (222). As would be expected, the depletion of MAdCAM-1 mirrors the effect of depleting the α4 integrin, providing significant protection from insulitis and disease development (216, 223).

The gut immune system plays a significant role in priming effector diabetes-inducing lymphocytes. Diabetogenic lymphocytes can be derived almost exclusively from the GALT of three week old NOD mice, while later in life diabetogenic lymphocytes can be derived from spleen, pancreatic lymph nodes and to a lesser extent from the GALT (224). This indicates that initial priming of the immune system could occur in the gut and that pancreatic lymph nodes can amplify the later response (224). It

has also been found that PBMCs depleted of $\alpha4\beta7^+$ lymphocytes isolated from T1D patients had a decreased response to GAD, indicating that the majority of autoantigen specific cells are associated with the GALT (218). Thus, it is likely that many of the lymphocytes responsible for insulitis and the development of diabetes are primed in the GALT and then home to the pancreas. The NOD.Itgb7$^{-/-}$ mice develop diabetes at a lower frequency than standard NOD mice, purportedly due to the inhibition of entry of lymphocytes into the islets. Antibody development to WP5212 appears to be dependent on integrin β7, indicating that priming of WP5212 reactive cells occurs in the GALT after which the activated cells could home to the pancreas and be involved in the inflammatory insulitic process via an unknown mechanism.

WP5212 antibodies, islet neogenesis and autoimmunity

In order to characterize the B cell antigenic epitopes of WP5212, an epitope library was constructed and immunoscreened with serum from the highly wheat-sensitive patient with both type 1 diabetes and celiac disease. It had previously been shown that this individual displayed high titer antibodies to WP5212 in comparison with control subjects (Figure 21, *panel A* and *B*). The library screening resulted in the identification of a 10 amino acid peptide that is predicted to form an α-helix on the three-dimensional structure of WP5212 (Figure 29 and 32).

The antigenic peptide shares sequence homology with the intermediate filament proteins desmin and vimentin (Figure 29; Table 9). Both these intermediate filament proteins have been associated with structures in the pancreas. Desmin is a marker of activated pancreatic stellate cells, which are involved in the development of fibrosis in

chronic, alcohol induced and autoimmune pancreatitis (225-227). Pancreatic stellate cells have also been associated with fibrosis in acinar tissue during diabetes development (228, 229). During an inflammatory process such as insulitis, immune mediators including interferon-γ, tumor necrosis factor α or production of nitric oxide can damage or destroy β-cells as well as neighboring cells (230). This 'bystander death' can result in the release of normally sequestered antigens from cells (230). It is possible that stellate cells present in the islets or in surrounding acinar area are being destroyed in this manner resulting in WP5212 cross-reactive antibody production to the stellate cell protein desmin.

The intermediate filament protein vimentin is considered to be a marker of the intermediate progenitor cell by which duct cells transdifferentiate into β cells during islet neogenesis (231, 232). During fetal islet formation, transient coexpression of vimentin and cytokeratin has been observed in duct cells but not in endocrine cells. However, this intermediate filament protein was not observed in duct epithelial cells after birth, suggesting that it is a marker for pancreatic islet progenitor cells (231). Also, among embryonic duct cells expressing vimentin, a small proportion coexpress PDX-1, a transcription factor associated with the differentiation of endocrine precursor cells (232). Vimentin intermediate filaments were also reported in proliferating duct cells in the embryonic and regenerating rat pancreas, as well as the pancreas of human type 2 diabetic patients further indicating that it is a marker of pancreatic precursor cells and dedifferentiation of fully matured pancreatic duct cells (232). After partial pancreatectomy, expression of vimentin in duct cells overlaps with duct cell proliferation (233). The coexpression of cytokeratin (epithelial cell marker) and vimentin (mesenchymal cell marker) intermediate filaments in the duct epithelium after partial

pancreatectomy could represent epithelial precursor cell activity during tissue regeneration in the postnatal period and thus could be considered a marker of dedifferentiation to an intermediate cell phenotype (232). These data indicate a role for vimentin in pancreas development and islet neogenesis. Antibody cross-reactivity between vimentin and WP5212 could indicate a link between wheat proteins and islet neogenesis. Therefore, the amino acid sequence homology between the WP5212 antigenic peptide and the intermediate filament proteins desmin and vimentin could represent molecular mimicry between an environmental agent and pancreatic structures.

To determine whether these proteins are cross-reactive with WP5212 antibodies, the same WP5212 antibody positive serum used for the initial epitope library screening was preabsorbed with desmin or vimentin and used to probe 1D Western blots of WP5212-baculovirus infected insect cell lysate. While desmin did not absorb WP5212 antibodies (Figure 33), serum preabsorbed with vimentin demonstrated decreased antibody binding to WP5212 (Figure 34). Therefore, WP5212 antibodies are cross-reactive with vimentin, presenting a case for molecular mimicry between a dietary antigen and a marker of islet neogenesis.

Based on these data, we propose that islet neogenesis could be a target of the autoimmune destructive process. Duct-like structures called pancreatic tubular complexes or neogenic foci are sites of islet neogenesis and are induced in response to pancreatic injury (234-236). Two recent studies demonstrated that tubular complexes are upregulated during the progression of autoimmune diabetes (Wang, Rosenberg and Scott, submitted) or under physiologically diabetes-like, hyperglycemic conditions (237). In both cases, these sites of islet neogenesis were infiltrated with mononuclear cells.

Inflammatory cells in and around the tubular complexes in BBdp rats suggests that β cells within these structures are targeted by an autoimmune attack as corroborated by the absence of insulin positive cells in tubular complexes in overt diabetic rats (Wang, Rosenberg and Scott, submitted). Therefore, immune activation by dietary WP5212 could result in cross-reactive immunity that is directed against vimentin positive areas of islet neogenesis.

Potential mechanisms of WP5212-related diabetes

Autoimmune diabetes results from the loss of immune tolerance to insulin producing β-cells in the pancreas. Molecular mimicry has been proposed as one of the mechanisms by which environmental factors initiate and/or accelerate a dysregulated immune response against self tissues. Molecular mimicry is defined as a structural similarity between two or more antigens that originate from self and foreign molecules (172). This can happen because individual B and T cells are degenerate, and thus cannot always distinguish between two molecules (238, 239). Therefore, if an immune response is activated in reaction to one molecule, for example a viral protein, immune tolerance to a similar (but not necessarily identical) self antigen can be lost and cross-reactive immunity occurs. The result can be damage to self tissues and disease. Although this mechanism of autoimmune initiation is attractive, there are no examples in which it has been proven in relation to spontaneous autoimmune diabetes.

Infectious agents have been linked to the development of a number of immune-mediated diseases, including type 1 diabetes. Viruses have been associated with T1D with the proposed mechanism being molecular mimicry (158). For example, insulin

autoantibodies from T1D patients have been shown to be cross-reactive with the retroviral antigen p73 (86). Also, a six residue amino acid homology (PEVKEK) was observed between a CBV protein and the autoantigen GAD, consistent with the hypothesis that CBV can induce diabetes via molecular mimicry.

Molecular mimicry has also been proposed as the mechanism by which diet-induced diabetes occurs. The ABBOS peptide is derived from bovine serum albumin (BSA) found in cow milk (240). During viral infections, the protein p69 is expressed on the surface of islet β-cells in response to an increase in the inflammatory cytokine IFNγ (240-243). p69 and the ABBOS peptide share a 17 residue antigenic epitope (240). The ABBOS peptide is proposed to induce an inflammatory response in susceptible individuals. Therefore, if a viral infection follows the induction of ABBOS immunity, then p69 becomes a target of the immune response upon its expression leading to immunity against insulin-producing β-cells (244). After the viral infection has subsided, p69 expression is down-regulated and can stop the autoimmune response (244). However, the relationship of the ABBOS peptide and BSA with diabetes has not been supported in subsequent studies (245) and remains controversial.

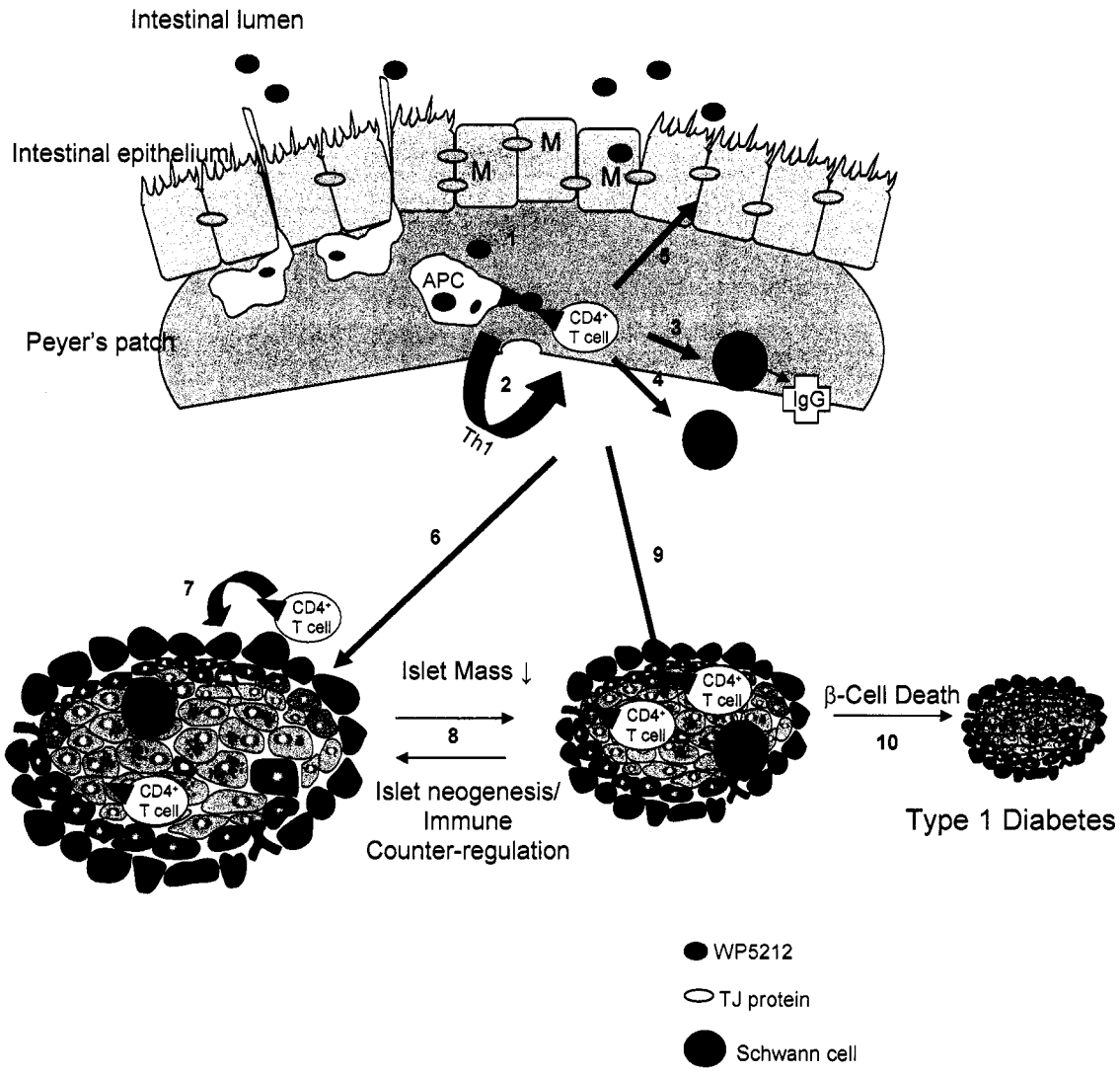
Molecular mimics are thought to contribute to the development of an autoimmune state by initiating or activating an immune response. In the context of dietary factors, the induction could begin in the gut associated lymphoid tissues, as this is the primary site of interaction between dietary antigens and the immune system (205). The intestine of diabetic individuals has been shown to be in a pro-inflammatory state, both before and after diabetes onset (128, 150, 151, 200, 246). This pre-existing inflammatory environment may allow for the aberrant activation of the immune response and the

subsequent loss of tolerance to dietary antigens. If the offending dietary antigen shares structural similarity with, and is therefore a molecular mimic of a β-cell specific molecule, then tolerance to β-cells will also be broken.

There are a number of points at which WP5212 could become involved in the initiation or promotion of islet autoimmunity. Firstly, the wheat protein WP5212 shares homology with a number of plant allergens from peanut, soybean and dandelion (Table 5). These molecules are known to be immunomodulatory, possibly by a shared mechanism due to the presence of structurally similar antigenic epitopes. WP5212 could stimulate gut-associated DCs to secrete IL-12 and IL-18, creating a cytokine microenvironment that promotes polarization of inflammatory T and B cells (Figure 35) (129). The result would be T cell production of IFNγ and B cell isotype switching to IgG. This general inflammatory immune response could allow for a decrease in the threshold of oral and self tolerance, creating an environment that promotes β-cell specific autoimmunity in susceptible individuals.

WP5212 shares homology with tight junction proteins, which form part of a protein complex that controls the permeability of the intestinal epithelium. WP5212 could induce an immune response that targets the tight junction proteins by cross-reactivity, followed by a decrease in mucosal barrier function. Indeed, it has been shown that diabetes-prone BB rats and type 1 diabetes patients have increased intestinal permeability (200, 201), possibly the result of an inflammatory mechanism (247). Following a breach in intestinal barrier integrity, the passage of dietary and pathogen antigens across the epithelium and their uptake by APCs residing in the PPs and LPs would increase. T cells trafficking

Figure 35. Possible mechanisms of WP5212-related diabetes. 1) WP5212 is taken up by gut-associated APCs in the Peyer's patch, 2) which stimulates them to secrete IL-12 and IL-18, that in turn promotes 3) B cell isotype switching to IgG and 4) polarization of inflammatory T cells. 5) Due to cross-reactivity between WP5212 and tight junction proteins, an inflammatory immune reaction could target and inhibit mucosal barrier function resulting in an increased dietary antigen load and decreased oral tolerance. 6) T cells activated in the GALT upregulate the expression of the integrin $\alpha 4\beta 7$, allowing them to traffic to the pancreas via interactions with its ligand MAdCAM-1, which is expressed on the exocrine pancreas and endothelial cells around the islets. 7) These lymphocytes attack the Schwann cells that envelope the islets by immune cross-reactivity between WP5212 and GFAP. 8) The immune-mediated attack and the subsequent decrease in islet mass stimulates compensatory islet neogenesis. 9) Vimentin positive duct-like precursor cells become a target of the immune reaction as a result of cross-reactivity with WP5212. 10) The balance between islet destruction and neogenesis can no longer be maintained resulting in a critical loss of β cells and consequently diabetes. APC, antigen presenting cell; M, microfold (M) cell; Th1, pro-inflammatory T helper 1; TJ, tight junction.


Intestinal lumen
Intestinal epithelium
M
M
M
1
APC
5
CD4+ T cell
3
Peyer's patch
2
4
IgG
Th1
6
9
7
CD4+ T cell
Islet Mass ↓
8
CD4+ T cell
CD4+ T cell
β-Cell Death
10
CD4+ T cell
Islet neogenesis/ Immune Counter-regulation
Type 1 Diabetes
WP5212
TJ protein
Schwann cell

through the GALT would have a greater chance of being activated resulting in the subsequent expression of the integrin α4β7. During the early stages of insulitis before diabetes onset, the expression of MAdCAM-1, the ligand for α4β7, is increased on vessels in the exocrine pancreas and on endothelial cells around the islets (216, 217). Therefore, T cells activated in the GALT could (and have been shown to) migrate into the pancreas. With increased numbers of activated T cells trafficking through the pancreas, the probability of islet autoimmunity would increase.

Another way in which environmental antigens could be involved in diabetes pathogenesis is by enhancing or accelerating an underlying, subclinical autoimmune process. The pancreas responds to injury, including autoimmunity, by initiating islet neogenesis (233, 248-252) (Wang, Rosenberg and Scott, submitted). Islet homeostasis may be disrupted when autoimmune destruction of β cells outstrips regenerative processes, such as neogenesis, until insufficient islet mass is left to maintain glucose homeostasis. WP5212 contains a number of peptides that are predicted to be T cell epitopes, specifically for the diabetes-related HLA-DR3 and DR4 molecules (Table 10 and 11). The predicted MHC class II peptides include the identified WP5212 B cell epitope, which shared homology with the self proteins vimentin and GFAP. Therefore, dietary WP5212 peptides could be taken up and presented by intestinal APCs to naïve T cells trafficking through the PPs, LP or MLNs. Their activation within the GALT would result in the upregulation of the integrin α4β7, allowing for their trafficking through the pancreas via the interaction between MAdCAM-1 and α4β7. The activated pro-inflammatory T cells could then target areas of islet neogenesis or, alternatively, pancreatic Schwann cells by cross-reactivity with vimentin or GFAP, respectively.

Therefore, WP5212 could induce or augment an autoimmune process by T and B cell molecular mimicry. β cell regeneration by islet neogenesis could no longer counter-balance β cell destruction, leading to a critical loss of islet mass. Also, immune-mediated destruction of the pancreatic Schwann cells could lead to diabetes development, as it is considered to be an integral part of the natural progression to diabetes (202).

CONCLUSION

Environmental agents have repeatedly been implicated in the pathogenesis of autoimmune diabetes. Of these environmental factors, diets containing wheat as the main or sole amino acid source have consistently resulted in the highest incidence of diabetes in both rodent models of spontaneous diabetes development, the BBdp rat and the NOD mouse. Despite substantial evidence implicating wheat in the development of diabetes, the specific diabetes-promoting component(s) have not previously been identified. Therefore, the primary goal of this project was to identify unique, diabetes-related wheat proteins.

Our hypothesis is that diabetes progression is dependent on dietary wheat proteins in a subset of susceptible individuals. Exposure to diabetes-promoting wheat proteins in these individuals activates an immune reaction that triggers or enhances islet autoimmunity and could also have direct or indirect effects on islet mass. We proposed to use this abnormal immune response as reflected by circulating IgG antibodies to identify T1D-related wheat proteins. Similar approaches have been used in the identification of all of the T1D-associated autoantigens, as well as numerous food allergens. We used pooled IgG antibodies from wheat-fed BBdp rats to screen a wheat cDNA expression library consisting of over 1 million recombinant wheat proteins. This is a novel approach, which permitted us to screen the proteome of the growing wheat endosperm.

Three candidate diabetes-related wheat proteins were initially identified. Upon screening the proteins with individual serum samples from BB rats at varying risk of diabetes development, it became apparent that antibody reactivity to one clone, WP5212,

correlated with diabetes risk. The results demonstrated not only that wheat protein-fed, diabetes-prone BB rats develop a strong immune reaction against the protein WP5212, but that the antibody reactivity was closely linked with the diabetogenic process in the target tissue. Additional studies revealed that WP5212 antibody reactivity was associated with diabetes in NOD mice, and importantly, in human patients with T1D. Of particular note, patients with both T1D and CD displayed high WP5212 antibody titers, possibly representing a marker of the propensity of T1D patients to develop CD.

The wheat storage globulin WP5212 shares homology with a number of other environmental and self proteins, from which many intriguing hypotheses can be formed. WP5212 shared amino acid sequence homology with food allergens suggesting that immunomodulatory dietary antigens could interact with the immune system via shared structures and mechanisms. Also, a number of cereals and other food plants contain proteins that shared sequence and structural homology with WP5212 presenting the possibility that diabetes-promoting proteins could exist in several plants. Furthermore, WP5212 shared homology with some self proteins, including intestinal tight junction proteins, protein markers of islet neogenesis and proteins associated with pancreatic Schwann cells. These homologies raise the possibility of immune cross-reactivity between WP5212 and these proteins, as routes for autoimmune destruction, which in turn could trigger or enhance the progression of diabetes.

This is the first candidate dietary wheat protein to be implicated in the development of autoimmune disease. Thus, screening a cDNA expression library provides a new approach that will enable others to identify novel environmental antigens involved in the progression of diabetes. The evidence presented in this thesis suggests

that WP5212, a Glb1 homologue, is strongly antigenic in three separate species that develop spontaneous diabetes. Having the molecular identity of an immune-targeted dietary wheat protein opens new avenues for the elucidation of the pathogenic mechanisms by which wheat-related diabetes occurs.

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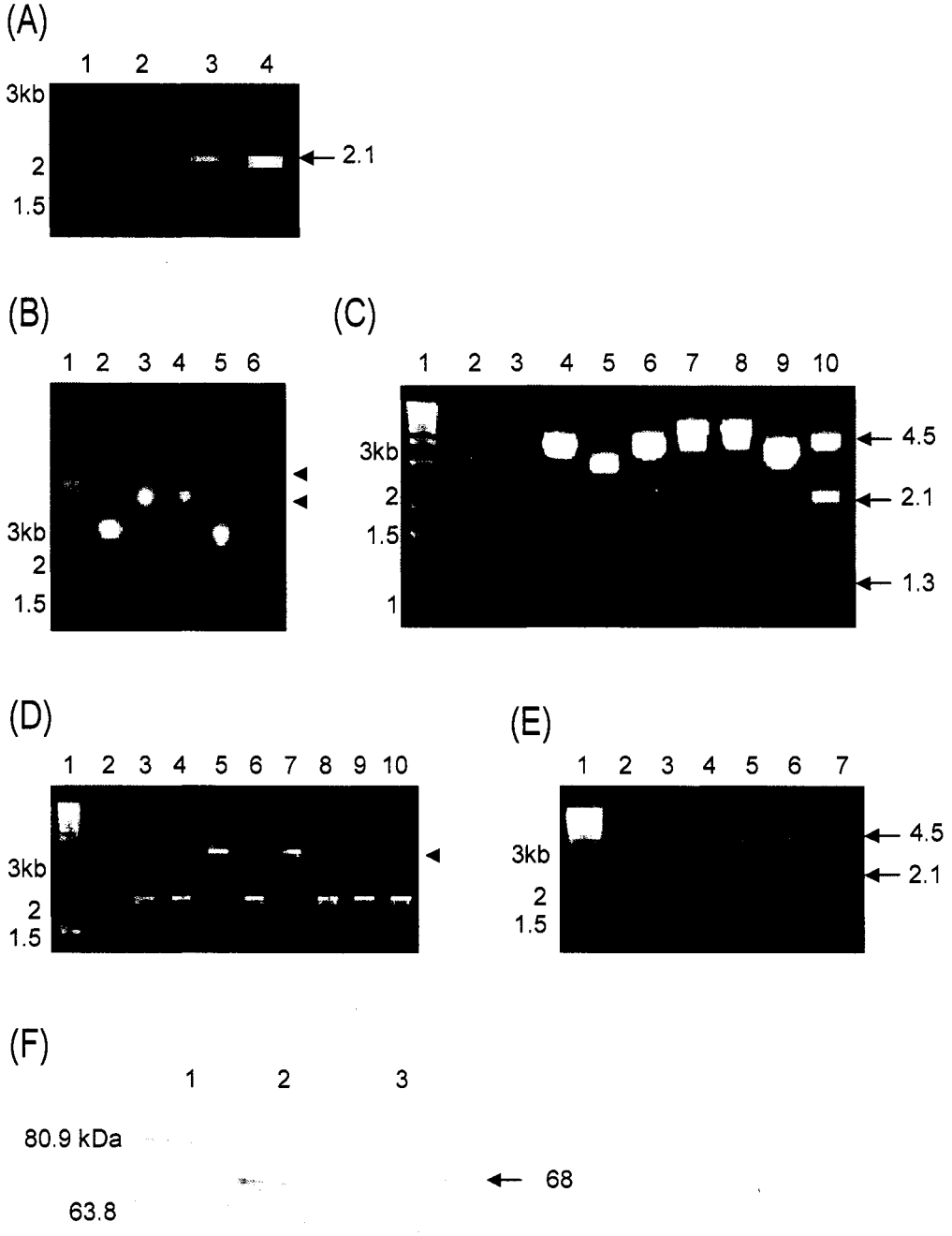
APPENDIX I: *E. coli* expression of recombinant WP5212

OBJECTIVE: To clone and express 6X His-tagged WP5212 for protein purification

MATERIALS AND METHODS

PCR amplification and subcloning WP5212 into the pET17b expression vector. The forward PCR primer was designed to include an *Nde* I restriction enzyme site, a start codon and a 6X His tag in frame with the WP5212 cDNA insert (5'-TAGTGACATATGCATCACCATCACCATCACATGGCGACCAGAGGCAGA-3'). The reverse primer was designed to be specific to the pBK-CMV phagemid sequence 3' of the wheat cDNA insert, including an *Xho* I site (5'-CGTCAGGCTCTAGAAGTACTC TCGAGTT-3'). Primers were purchased from Sigma Genosys (Mississauga, ON). The WP5212 sequence was amplified by PCR. The 2.1 kb WP5212 PCR product (Figure A1, *panel A*) was cloned into the pCR2.1 plasmid using TOPO TA-cloning® according to the manufacturer's instructions (Invitrogen, Carlsbad, CA; Figure A1, *panel B*). Plasmid DNA from clones containing inserts were prepared according to the manufacturer's instructions (Promega, Madison WI). WP5212 was digested with *Nde* I and *Xho* I (Figure A1, *panel C*) and cloned into the *Nde* I/*Xho* I restriction enzyme site of the pET17b expression vector (Novagen, Madison, WI; Figure A1, *panel D*) following the instructions provided with T4 DNA ligase (Invitrogen). The presence of WP5212 was confirmed by restriction digestion with *Nde* I and *Xho* I (Figure A1, *panel E*) and sequencing performed by the University of Ottawa Biotechnology Research Institute.

Figure A1. Construction of a His-tagged WP5212 pET17b expression vector. *Panel A*, PCR amplification of the WP5212 cDNA sequence (Lane 1, 1kb Plus DNA ladder; lane 2 negative control; lane 3, positive control; lane 4, WP5212). *Panel B*, plasmid minipreps of WP5212-pCR2.1 ligation products (Lane 1, 1kb Plus DNA ladder; lanes 2-6, WP5212-pCR2.1 clones 1-5). Clones 2, 3 and 5 have shifted up indicating insertion of the WP5212 PCR product. *Panel C*, confirmation of ligation by *Nde* I/*Xho* I restriction digest (Lane 1, 1kb Plus DNA ladder; lane 2, negative control; lane 3, pET17b digested with *Nde* I only; lane 4, WP5212-pCR2.1 clone 1 digested with *Xho* I only; lane 5, uncut WP5212-pCR2.1 clone 1; lanes 6-10, WP5212-pCR2.1 clones 1-5 digested with *Nde* I and *Xho* I). WP5212-pCR2.1 clones 2, 3 and 5 have 1.3, 1.3 and 2.1 kb, respectively. WP5212-pCR2.1 clone 5 has the full insert, as confirmed by sequencing. WP5212-pCR2.1 clones 2 and 3 have truncated inserts of the 5' end of WP5212, as confirmed by sequencing. *Panel D*, plasmid miniprep of WP5212-pET17b ligation products (Lane 1, 1kb Plus DNA ladder; lane 2-10, WP5212-pET17b clones 1-9). WP5212-pET17b clones 4 and 6 have shifted up indicating insertion of the WP5212 gene. *Panel E*, confirmation of ligation by *Nde* I/*Xho* I restriction digest (Lane 1, 1kb Plus DNA ladder; lane 2 uncut pET17b; lane 3, pET17b digested with *Nde* I only; lane 4, pET17b cut with *Xho* I only; lanes 5-7, WP5212-pET17b clones 3, 4 and 6 digested with *Nde* I and *Xho* I). WP5212-pET17b clones 4 and 6 have 2.1 kb inserts correlating with the expected size of the WP5212 insert. *Panel F*, IPTG-induced expression of WP5212 in BL21(DE3)pLysS *E. coli*. SDS-PAGE and Western blot analysis of samples before and after 4 hs of IPTG-induction (Lane 1, Benchmark Prestained Protein Ladder (Invitrogen); lane 2, 4 hr after IPTG-induction; and lane 3 pre-IPTG-induction).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3kb
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1 2 3 4 5 6 7 8 9 10
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(D)
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(E)
1 2 3 4 5 6 7
3kb
2
1.5
4.5
2.1
(F)
1 2 3
80.9 kDa
63.8
68

His-tagged 5212 protein expression in E. coli. A 500 ml culture of recombinant 6X His-WP5212/pET17b was inoculated with 25 ml of an overnight culture and incubated with shaking at 37°C until OD_{600} = 0.3-0.4. Protein expression was induced with the addition of IPTG to a final concentration of 1 mM. Samples were taken after 1, 2, 3 and 4 hs of induction. The culture was centrifuged and the pellet was resuspended in protein lysis buffer (per 50 ml: 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0, protease inhibitor cocktail tablet (Roche, Mannheim, Germany), 1 mM PMSF, 10 mM β-mercaptoethanol, 1 mg/ml lysozyme). The supernatant (soluble protein fraction) was transferred to a new tube and the pellet was resuspended in insoluble protein fraction urea buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0, 10 mM β-mercaptoethanol, 8 M urea).

1D SDS-PAGE and Western Blotting of WP5212. 1D SDS-PAGE and Western blotting were performed as previously described. Membranes were probed with mouse anti-His_6 (Roche) diluted to 0.2 μg/ml in blocking buffer. The secondary antibody was goat anti-mouse polyvalent immunoglobulins conjugated to alkaline phosphatase (Sigma) diluted 1:10,000 in blocking buffer. Antibody binding was detected using alkaline phosphatase developer containing NBT (0.3 mg/ml) and BCIP (0.15 mg/ml).

In vitro *transcription and translation of His-tagged 5212.* Biotinylated WP5212 protein was expressed using the TNT T3 Coupled Reticulocyte Lysate System (Promega, Madison WI) according to the manufacturer's instructions. Non-radioactive Transcend™ biotinylated lysine tRNA (Promega) was added to the reaction to label the expressed protein. The *in vitro* transcription and translation samples were used for 1D SDS-PAGE and Western blotting. The biotinylated proteins were incubated with streptavidin

conjugated to alkaline phosphatase (Promega) and developed in Western Blue Stabilized Substrate for AP (Promega), according to the manufacturer's instructions.

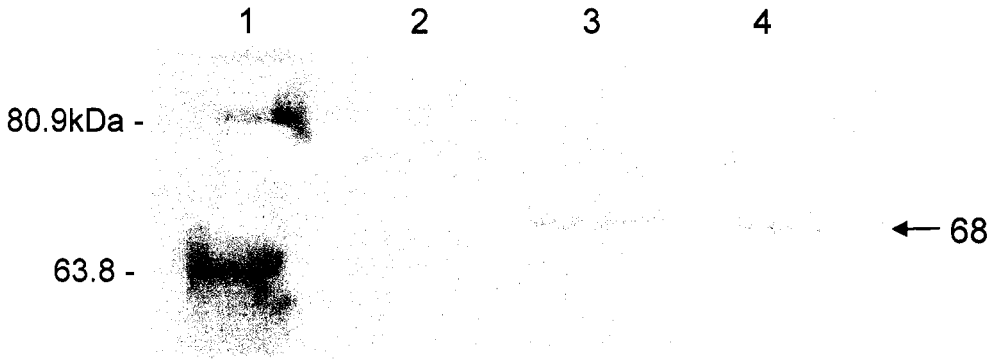
RESULTS AND DISCUSSION

A 6x His-WP5212/pET17b expression construct was successfully constructed as determined by restriction digestion (Figure A1, *panel E*) and sequencing of the vector. However, only minimal WP5212 protein expression was achieved. The level of expression did not exceed that observed prior to induction with IPTG (Figure A1, *panel F*). Expression could not be upregulated by altering IPTG concentration, culture OD value at IPTG induction or length of IPTG induction (data not shown). Also, the level of 6X His-tagged WP5212 expression could not be increased by transforming different *E. coli* strains (XLOLR, XL1-Blue-MRF', BL21-Gold, BL21-Gold(DE3), BL21-Gold(DE3)pLysS, BL21-CodonPlus (DE3)-RP (all from Stratagene), data not shown) with the 6x His-WP5212/pET17b expression construct. Furthermore, the concentration of 6x His-WP5212/pET17b plasmid isolated from all *E. coli* strains was low in comparison to all other wheat clones, as well as expected values for the plasmid preparation kits. For example, 6X His-WP5212/pET17b yielded a total of 45 μg of plasmid DNA (data from May 29, 2003; using Wizard Plus Maxiprep DNA purification System, Promega). The expected plasmid yield for a high copy plasmid is at least 500 μg (according to the manufacturer's protocol). There appears to be selection for low expression and low plasmid copy, suggesting that the WP5212 protein is toxic to *E. coli*.

In vitro transcription and translation of 6X His-tagged WP5212 was used to by-pass the issue of toxicity in *E. coli*. Although rabbit reticulocyte *in vitro* transcription and

translation was successful using the 6X His-WP5212/pET17b construct (Figure A2), it was not possible to scale-up protein production with this method. Therefore, *in vitro* transcription and translation using a cell-free protein expression system (Rapid Translation System; Roche) was attempted, as this system allows for both small and large scale expression. However, it too was unsuccessful (data not shown).

Figure A2. *In vitro* transcription and translation of the His-tagged WP5212 pET17b expression vector. Western blot analysis of alkaline phosphatase labeled 6X-His-tagged WP5212 expressed in a rabbit reticulocyte lysate *in vitro* transcription and translation reaction (Lane 1, Benchmark Prestained Protein Ladder (Invitrogen); lane 2, negative control; lanes 3 and 4, 6X-His-tagged WP5212 clones 1 and 2). A positive control reaction expressing luciferase was performed (data not shown). Luciferase chemiluminescence activity was detected using the Kodak Image Station.


1
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4
80.9kDa -
63.8 -
68

APPENDIX II: Probing islets with WP5212 polyclonal antibodies in the search of diabetes-associated self proteins

OBJECTIVE: To identify diet-specific WP5212 antibody cross-reactive proteins in isolated islets from BBdp rats.

MATERIALS AND METHODS

Pancreatic islet isolation. Islets of Langerhans were isolated from 30 d BBdp rats fed either the NTP or HC diet from weaning by Dr. Liisa Kauri in the Scott lab. Islets were isolated from rat pancreata using ductal injection of collagenase buffer (1.5 mg/mL; Sigma) as described previously (253-255).

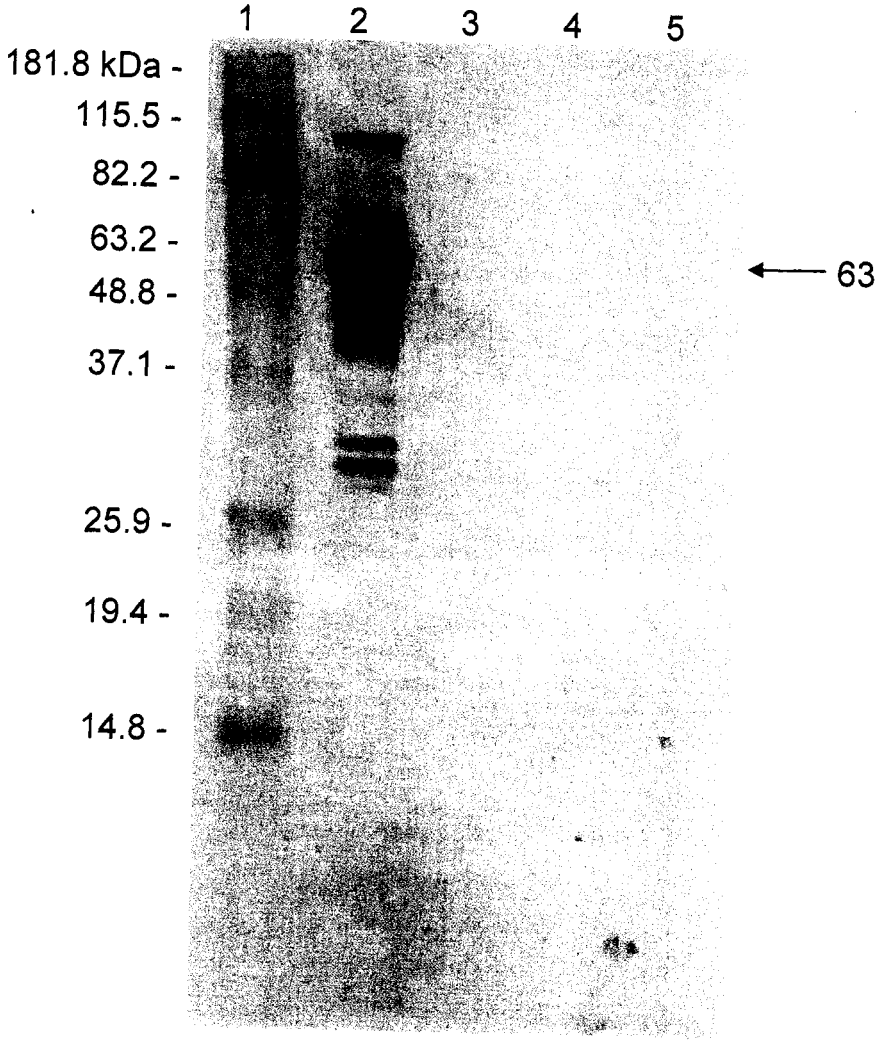
1D SDS-PAGE and Western Blotting of isolated islets. 1D SDS-PAGE and Western blotting were performed as previously described. The islet samples were solubilized in 1X SDS-PAGE loading buffer. WP5212-baculovirus infected Sf21 insect cell lysate was used as a positive control. Membranes were probed with polyclonal rabbit anti-WP5212 serum (from above). The secondary antibody was goat anti-rabbit immunoglobulins conjugated to alkaline phosphatase (Dako Diagnostics) diluted 1:1000 in blocking buffer. Antibody binding was detected using alkaline phosphatase developer containing NBT (0.3 mg/ml) and BCIP (0.15 mg/ml).

RESULTS AND DISCUSSION

The polyclonal rabbit anti-WP5212 serum was used to probe Western blots of isolated islets to identify potential WP5212 cross-reactive islet proteins. However, no

proteins were bound by WP5212 specific antibodies. The antisera was produced in rabbits co-immunized with two WP5212 peptides, as described above, rather than the full length protein. Therefore, islet proteins could cross-react with other epitopes from WP5212.

Figure A3. Western blot analysis of isolated BBdp rat islets screened with the polyclonal rabbit WP5212 antibodies. Pancreatic islets were isolated from 30d BBdp rats fed the NTP or HC diet (lanes 4 and 5), and from 30d BBc rats fed the NTP diet (lane 3). 1D SDS-PAGE and Western blotting was performed with total islet protein. The Western blots were probed with rabbit anti-WP5212 polyclonal antibodies for detection of cross-reactive islet proteins. WP5212-baculovirus infected insect cell lysate (10 μg protein, lane 2) was used as a positive control. Lane 1 contains the Benchmark prestained ladder.


1
2
3
4
5
181.8 kDa -
115.5 -
82.2 -
63.2 -
48.8 -
37.1 -
25.9 -
19.4 -
14.8 -
63

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EDUCATION

2000 - 2004 University of Ottawa

Ph.D. in Biochemistry

- Thesis: Identification and characterization of the type 1 diabetes-related wheat storage globulin WP5212

1996 - 2000 Carleton University

B.Sc. Highest Honours in Biology and Biotechnology

- Thesis: *In vivo* photostabilization of tetracycline hydrochloride by reduced glutathione for use as an ecological marker in *Salmo salar* (Atlantic salmon)

WORK EXPERIENCE

2000 – present Ottawa Health Research Institute

Ph.D. candidate. Identification and characterization of diabetes-related wheat antigens using techniques in molecular biology

2001 – present Carleton University

Residence manager. Responsible for the direct supervision of four residence fellows and the behaviour, health and well-being of over 210 undergraduate students

2002 Health Products and Food Branch, Health Canada

Instructor. Topics in Molecular Biology/Biotechnology Series for the Continuing Education & Training Program

2001 Faculty of Medicine, University of Ottawa

Lecturer. Hypersensitivity and autoimmune disorders workshop for medical students

2000 – 2001 Kaplan (Canada) Inc.

Instructor. SAT preparatory course

2000 Department of Biology, Carleton University

Leader. "Art Under the Microscope" for the Carleton University Experience high school program

1999 – 2000 Ottawa-Carleton School Board

Teacher's assistant. Grade 7 and 8 math and science

1998-2000 Department of Biology, Carleton University

Leader. Carleton University Partner for the Holy Trinity High School Biology Independent Study Program

PUBLICATIONS

Chakir, H., **A.J. MacFarlane**, D. Lefebvre, M. Mojibian, J.R. Webb, C. Touchie, J. Karsh, F.W. Scott. A highly wheat sensitive patient with type 1 diabetes, celiac disease and antibodies to Glb1. *In preparation.*

MacFarlane A.J., K.M. Burghardt, J. Kelly, T. Simell, O. Simell, I. Altosaar, F.W. Scott. A type 1 diabetes-related protein from wheat (*Triticum aestivum*); cDNA clone of a wheat storage globulin, Glb1, linked to islet damage. Journal of Biological Chemistry *278* (1): 54-63. 2003.

MacFarlane, A.J., and F.W. Scott. Environmental agents and type 1 diabetes. In, *Textbook of diabetes, third edition*. Pickup, J.C., and G. Williams, eds. Blackwell Science Ltd Oxford UK. 2003.

MacFarlane, A.J., D.L. Sutherland, T.R. Goff and K.M. Gilmour. *In vivo* photostabilization of tetracycline hydrochloride by reduced glutathione for use as an ecological marker in *Salmo salar*. Aquaculture *213* (1-4): 253-263. 2002.

PRESENTATIONS AT SCIENTIFIC MEETINGS

06/2004 American Diabetes Association 64th Scientific Sessions, Orlando, Florida
Oral presentation: Epitope mapping of the diabetes-associated wheat storage globulin, Glb1. **MacFarlane, A.J.**, and F.W. Scott

04/2004 Experimental Biology 2004 Annual Meeting, Washington, D.C.
Poster presentation: Prediabetic NOD mice have increased antibodies to Glb1, a wheat storage globulin. **MacFarlane, A.J.**, and F.W. Scott

01/2004 Plant and Animal Genome Conference XII, San Diego, California
Poster presentation: Wheat (*Triticum aestivum*) genes and type 1 diabetes. McNulty, M.S., E. Loit, M.A. Zaidi, **A.J. MacFarlane**, F.W. Scott, S. Postel and I. Altosaar

12/2003 3rd Annual Ottawa Health Research Institute Research Day, Ottawa, ON
Oral presentation: Wheat storage globulin, Glb-1: A type 1 diabetes-related antigen. **MacFarlane, A.J.**, and F.W. Scott

03/2003 JDRFI Meeting/Workshop: Gut dysfunction, diet & autoimmune diabetes, Montebello, QC
Oral presentation: Glb1 and diabetes risk: Not only the BB rat? **MacFarlane, A.J.**, and F.W. Scott

06/2002 11th International Congress of Mucosal Immunology, Orlando, Florida
Poster presentation: Diabetes-prone BB rats fed a wheat gluten diet develop IgG antibodies to a cloned wheat storage globulin. **MacFarlane, A.J.**, and F.W. Scott

09/2001 37th Annual Meeting of the European Association for the Study of Diabetes, Glasgow, Scotland
Poster presentation: Immunoscreening a wheat cDNA library for candidate peptides involved in the pathogenesis of type 1 diabetes. **MacFarlane, A.J.**, K.M. Burghardt, R. Sardana, I. Altosaar and F.W. Scott

09/2001 JDRFI Meeting/Workshop: Gut dysfunction, diet and autoimmune diabetes, Glasgow, Scotland
Oral presentation: Immunogenic wheat peptides in type 1 diabetes. **MacFarlane, A.J.**, K.M. Burghardt, and F.W. Scott

06/2001 44th Annual Meeting of the Canadian Federation of Biological Societies, Ottawa, ON
Poster presentation: Screening a wheat cDNA library for immunogenic peptides involved in the pathogenesis of type 1 diabetes. **MacFarlane, A.J.**, K.M. Burghardt, R. Sardana, I. Altosaar and F.W. Scott

05/2001 JDRFI Group Meeting/Workshop: Gut dysfunction, diet and autoimmune diabetes, Brussels, Belgium
Oral presentation: In search of diabetogenic wheat peptides: Using cDNA expression library screening.

MacFarlane, A.J., K.M. Burghardt, R. Sardana, I. Altosaar and F.W. Scott

05/2000 39th Annual Meeting of the Canadian Society of Zoologists, St. Andrew's, NB
Poster presentation: Tetracycline as a marker in Atlantic salmon. **MacFarlane, A.J.**, D.L. Sutherland, T.R. Goff and K.M. Gilmour

03/2000 13th Annual Ontario Biology Honours Conference, Peterborough, ON
Oral presentation: Photostabilization of tetracycline hydrochloride by reduced glutathione for use as an ecological marker in *Salmo salar* (Atlantic salmon). **MacFarlane, A.J.**, D.L. Sutherland, T.R. Goff and K.M. Gilmour

PATENTS

2004	New Canadian and United States Patent Applications: *Diabetogenic Epitopes* Inventors: Fraser W. Scott, Amanda J. MacFarlane and Karolina M. Burghardt

HONOURS AND AWARDS

2001-2005	Ontario Ministry of Training, Colleges and Universities *Ontario Graduate Scholarship ($15,000 per annum)*
2001-2004	University of Ottawa *University of Ottawa Excellence Scholarship ($5,235 per annum)*
2004	University of Ottawa *Department of Biochemistry Travel Grant ($1,000)*
2004	University of Ottawa *Faculty of Graduate and Postdoctoral Studies Travel Grant ($250)*
2003	Juvenile Diabetes Research Foundation *Ron Oelbaum Diabetes Research Award for Outstanding Canadian Research Scientist under 35 ($2,000)*
2002 & 2003	University of Ottawa *Strategic Areas of Development Award ($6,000 per annum)*
2002	University of Ottawa *Admission Scholarship - Ph.D. Graduate Studies ($10,860)*
2002	University of Ottawa *Faculty of Graduate and Postdoctoral Studies Travel Grant ($400)*
2001	University of Ottawa *Department of Biochemistry Travel Grant ($1,000)*
2000	Carleton University *NSERC Undergraduate Student Research Award (declined) ($4,500)*
2000	Carleton University *Dean's list*
2000	Canadian Millennium Scholarship Foundation *Canadian Millennium Scholarship ($3,000)*
1996	Carleton University *Carleton University Academic Award ($500)*

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Dr. Karolina Burghardt
Formerly of the lab of Fraser Scott
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Ottawa, Ontario, Canada

- Dr. Karolina Burghardt isolated total RNA from hard red spring wheat, AC Barrie caryopses.

Dr. Illimar Altosaar
Department of Biochemistry, Microbiology and Immunology
University of Ottawa
Ottawa, Ontario, Canada

- Dr. Illimar Altosaar arranged through Dr. Vern Burrows of Agriculture and Agri-Food Canada to obtain hard red spring wheat caryopses from which RNA was isolated.

Dr. Markus Walz and Dr. Hubert Kolb
German Diabetes Research Institute
Heinrich Heine University of Düsseldorf
Düsseldorf, Germany

- We provided Dr. Markus Walz with the WP5212 phagemid for cloning into baculovirus.

Drs. Liisa Kauri and Habiba Chakir, and David Lefebvre and Majid Mojibian
Lab of Fraser Scott
Ottawa Health Research Institute
Ottawa, Ontario, Canada

- Dr. Kauri provided isolated BBdp rat islets.
- Dr. Chakir, Mr. Lefebvre and Mr. Mojibian isolated PBMCs from human study subjects for HLA typing.

Dr. Ed Leiter
The Jackson Laboratory
Bar Harbor, Maine, USA

- Dr. Leiter provided serum samples from 70 d NOR, NOD.Itgb7$^{-/-}$ and NOD.Itgb7$^{-/-}$ / L-sel$^{-/-}$ female mice.

Dr. Claire Touchie, Dr. Jacob Karsh and Dr. Erin Keely
Department of Medicine
Ottawa Hospital
Ottawa, Ontario, Canada

- Drs. Touchie and Karsh recruited the case study patient and Dr. Keely recruited patients for the human study.

Dr. Sarah Lawrence
Division of Endocrinology
Children's Hospital of Eastern Ontario
Ottawa, Ontario, Canada

- Dr. Lawrence recruited patients for the human study.

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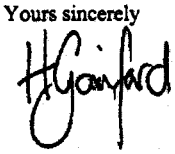

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