A diabetes-specific HLA-DR restricted pro-inflammatory T cell response to wheat polypeptides in tissue transglutaminase antibody negative patients with type 1 diabetes

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Additional information for this article can be found in an online appendix at http://diabetes.diabetesjournals.org

Submitted 20-April-2009 and Accepted 20-April-2009

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Objective: There is evidence of gut barrier and immune system dysfunction in some patients with type 1 diabetes, possibly linked with exposure to dietary wheat polypeptides (WP). However, the frequency of abnormal immune responses to wheat, their nature and whether such responses are diabetes-specific remains unclear.

Research design and methods: In type 1 diabetes patients and healthy control subjects, the immune response of peripheral CD3+ T cells to WP, ovalbumin, gliadin, α-gliadin 33-mer peptide, tetanus toxoid and PHA was measured using a CFSE proliferation assay. Th1, Th2 and Th17 cytokines were analyzed in WP-stimulated PBMC supernatants and HLA was analyzed by PCR.

Results: Twenty out of 42 patients displayed increased CD3+ T cell proliferation to WP and were classified as responders; proliferative responses to other dietary antigens were less pronounced. WP-stimulated PBMC of patients showed a mixed pro-inflammatory cytokine response with large amounts of IFN-γ, IL-17A and increased TNF. HLA-DQ2, the major celiac disease risk gene was not significantly different. Nearly all responders carried the diabetes risk gene, HLA-DR4. Anti-DR antibodies blocked the WP response and inhibited secretion of Th1 and Th17 cytokines. High amounts of WP-stimulated IL-6 were not blocked.

Conclusions: T cell reactivity to WP was frequently present in type 1 diabetes patients and associated with HLA-DR4 but not HLA-DQ2. The presence of an HLA-DR restricted, Th1 and Th17 response to wheat polypeptides in a subset of patients indicates a diabetes-related inflammatory state in the gut immune tissues associated with defective oral tolerance and possibly gut barrier dysfunction.
The gastrointestinal tract contains the largest collection of immune cells in the body. In healthy individuals, the gut immune system does not normally mount an immune response against molecules from foods and commensal bacteria, preferring a default state of immune unresponsiveness called oral tolerance [1].

When oral tolerance is broken, an immune imbalance results that can lead to increased gut permeability, inflammation and tissue damage. The best understood example of this is celiac disease, which is the classic food-induced autoimmune disorder and the only autoimmune disease for which the autoantigen, (tissue transglutaminase) and the inciting environmental factors (gluten proteins) are known [2]. In celiac disease, specific wheat gliadin peptides undergo deamidation by gut epithelial cell tissue transglutaminase and are presented to T cells on HLA-DQ2 or HLA-DQ8 molecules resulting in the stimulation of a Th1-biased pro-inflammatory attack that causes villous atrophy [2]. It has also been proposed that the gut and dietary antigens play an important role in human type 1 diabetes [3] based on animal studies, epidemiological reports and a small number of studies on human tissue (Reviewed in [4,5]).

The gut barrier and immune system in diabetes-prone rodents display abnormalities similar to celiac disease. For example, there are signs of enteropathy in diabetes-prone BB (BBdp) rats [6] and non-obese-diabetic (NOD) mice [7], inflammatory cytokines in the gut are increased [8,9], as is permeability before islet inflammation [10-12]. Closing gut tight junctions prevents diabetes in the rat [12], there is increased antibody and T cell response to dietary antigens [13,14] and wheat-based diets are major promoters of diabetes in rats and mice [4]. Diabetes appearance can be partly inhibited by early neonatal feeding of small amounts of wheat proteins to diabetes-prone BB rats by dampening the pro-inflammatory state of the gut [8]. There are also indications that a gluten-free diet can enhance islet mass in BB rats [15]. High risk children on a gluten-free diet for 6 months showed enhanced first phase insulin response during an I.V. glucose tolerance test, which could be an indication of increased β-cell mass and/or function [16,17]. Thus, wheat is one external factor that could influence the development of diabetes.

Normal regulation of the gut immune system depends on maintaining the integrity of the gut barrier [18]. There are now several reports of gut inflammation, and signs of gut damage or leakage in humans with type 1 diabetes [19-24]. T cells from human diabetic pancreas display gut mucosal homing properties [25] and T cells reactive against the diabetes autoantigen, GAD, express the gut-associated homing receptor α4β7-integrin [26]. In two prospective analyses of high risk children, early exposure to cereals including wheat increased the risk of islet autoimmunity [27,28]. Another study showed increased T cell proliferation in response to high concentrations of wheat gluten in 24% of patients [29]. Auricchio et al. reported inflammation and increased immune response to gliadin in jejunal biopsies from patients [30]. Approximately 2-6% of patients with type 1 diabetes have celiac disease, a rate that is several times higher than in the general population, and a recent report indicated that celiac disease patients on a gluten-free diet were protected from later development of type 1 diabetes [31]. These findings point to both a loss of barrier integrity and dysregulation in the gut immune system.

Thus, questions arise regarding the frequency of abnormal immune responses to wheat, their nature and whether such responses are diabetes-related, reflect a separate gut dysfunction, or are simply due to shared celiac risk genes, such as HLA-DQ2, -
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DQ8 and -DR3. The number of studies examining the response of type 1 diabetes patient immune cells to wheat proteins is limited [20,29,30] and it remains unclear whether there is a genetically determined abnormal immune response to wheat in humans at risk for type 1 diabetes. We favor the view that there is a diabetes-specific abnormal immune response to wheat in some patients that is not explained by shared celiac disease risk genes.

We hypothesize that in some type 1 diabetes patients, excessive amounts of wheat proteins/polypeptides enter the body through a leaky gut barrier and promote an abnormal immune response that breaks oral tolerance and stimulates immune cells that are involved in type 1 diabetes pathogenesis [4]. As a first step, it is important to clarify what proportion of patients with type 1 diabetes display abnormal immune reactivity to dietary wheat polypeptides and to characterize this abnormal response. The objectives of the present study were to determine whether patients with type 1 diabetes display increased T cell proliferation in response to a mixture of wheat polypeptides, to analyze the pattern of cytokines produced, and to determine whether this reactivity is associated with specific HLA alleles.

RESEARCH DESIGN AND METHODS

Subjects: Type 1 diabetes patients were recruited through physicians at the Ottawa Hospital and the Children’s Hospital of Eastern Ontario, Ottawa, Canada. All patients have clinically proven type 1 diabetes. The majority of the 42 subjects were young adults of both sexes with one child included. A total of 22 unrelated controls without acute infection or autoimmune disease were recruited in a similar age range and ethnic group (Caucasian) (Table 1). All individuals included in the study were negative for tissue transglutaminase antibody as measured by the Ottawa Hospital Clinical Laboratory. Blood was obtained by venepuncture from patients and healthy controls with informed consent. The local ethics committees approved the study.

Isolation of mononuclear cells, tissue culture and antigen response: Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation over Ficoll (Histopaque-1.077; Sigma-Aldrich, Oakville, ON, Canada). Cells were washed twice with Hank’s buffer (Invitrogen, Burlington, ON, Canada) containing 20 mM HEPES (Invitrogen). 20×10^6/ml PBMC were labeled with 2 mM CFSE (Invitrogen) for 20 minutes and incubated at 37°C in 5% CO₂. Cells were washed twice with Hank’s buffer containing 5% pooled human AB⁺ serum (Bioreclamation Inc., Hicksville, NY, USA) and finally diluted in RPMI-1640 (Sigma-Aldrich) containing 5% Human AB⁺ serum, 2 mM L-glutamine (Invitrogen), 25 mM HEPES (Invitrogen), 50 µM β-mercaptoethanol (Sigma-Aldrich) and 1% antibiotic/antimycotic (Invitrogen). Cells were cultured with various concentrations of wheat protein/peptides (chymotrypsin-treated, heat-inactivated wheat gluten, WP, ICN Biochemicals, Cleveland, OH, USA [32]) (3.1-12.4 µg/ml), gliadin 10 µg/ml (Sigma-Aldrich), α-gliadin 33 mer 10 µg/ml (a generous gift from Dr. Chaitan Khosla, Stanford University, Stanford, CA and Dr. Hubert Kolb, German Diabetes Center, Düsseldorf, Germany), insulin 10 µg/ml (Sigma-Aldrich), ovalbumin (OVA; Sigma-Aldrich) 1 µmol, Tetanus toxoid (TT, Connaught Laboratory, Toronto, ON, Canada) 2.7 LF/ml or PHA 5 µg/ml (Sigma-Aldrich). 1.2×10⁶ cells in 1 ml/well were cultured in 24 well plates (Falcon; VWR, Mississauga, ON, Canada). After 3 days of culture, 10 IU/ml rhIL-2 (PeproTech Inc, Rocky Hill, NJ, USA) was added to each well. On day 8, supernatants were harvested and cell
proliferation was assessed using a CFSE-based, flow cytometric assay with results expressed as cell division index (CDI; defined as the number of CD3⁺ CFSE^dim cells cultured with antigen/ number of CD3⁺ CFSE^dim cells without antigen; the number of CFSE^dim events was the number corresponding to 5000 CFSE^bright cells) [33].

Wheat protein preparations were analyzed for LPS. The concentration was low and comparable to other recombinant proteins. The addition of the LPS inhibitor polymyxin B (Sigma) had no effect on WP-induced T cell proliferation but blocked LPS-induced T cell proliferation (see Supplemental Fig. S1 in the online appendix available at http://diabetes.diabetesjournals.org).

**Blocking of HLA-DR with anti-DR antibody:** Inhibition of the proliferation of PBMCs to wheat protein was studied by adding monoclonal anti-DR antibodies (clone G46-6, mouse IgG2aκ, BD Biosciences, Mississauga, ON, Canada) to the culture 30 min before adding wheat protein to the cell culture wells. The mouse IgG2aκ isotype antibody (BD Biosciences) was used as a control. Both antibodies were used at a concentration of 5 µg/ml.

**Cytokine evaluation in culture supernatants:** Cytokines including IL-4, IL-6, IL-10, TNF and IFN-γ were quantified simultaneously using a human Th1/Th2 cytokine cytometric bead array (CBA) kit. The CBA kit and CBA software were purchased from BD Biosciences Mississauga, ON, Canada. All assays were performed according to the manufacturer’s protocol and samples were read in a Coulter FC500 flow cytometer after appropriate calibration. Quantification of cytokine levels was performed by comparison with standards provided in the kit using CBA software. IL-17A was analyzed in supernatants of PBMC from a subset of patients and healthy controls in the presence or absence of anti-DR antibodies using an ELISA kit purchased from eBioscience (San Francisco, CA, USA).

**HLA typing:** HLA-DR and DQ haplotypes of subjects were characterized by PCR-based HLA class II tissue typing using low resolution Olerup SSP™-typing kits for HLA-DR and DQ (Genovision Inc., West Chester, PA, USA) or by the Clinical Laboratory at the Ottawa Hospital.

**Statistical analysis:** Differences in CDI among patients and controls were analyzed by the non-parametric Mann-Whitney U-test, using GraphPad Prism version 4.03 for Windows, (GraphPad Software, San Diego, CA, USA). Significance of differences in frequencies was evaluated using Fisher’s exact two-tailed test. Spearman’s correlation was used to analyze the correlation between WP T cell response and the other dietary or autoantigens using STATISTICA version 6 (Statsoft, Tulsa, OK, USA).

**RESULTS**

**CD3⁺ T cell response to wheat polypeptides in control and type 1 diabetic subjects:** The CFSE assay permitted evaluation of T cell proliferation to low, non-toxic concentrations of WP (Fig. 1). CD3⁺ T cell proliferation in the presence of 3.1 and 6.2 µg/ml WP was significantly higher in the type 1 diabetes patient group compared with controls (Fig. 1). At 12.4 µg/ml, CDI was still higher in the type 1 diabetes group but the response was less than at 6.2 µg/ml suggesting inhibition. Therefore, 6.2 µg/ml was chosen as the optimum concentration. To identify patients with a positive proliferation response to WP, the mean +3 SD of the control group CDI (CDI ≥ 14.6) was chosen as the cut-off. Twenty of the 42 patients with type 1 diabetes (47%) had a positive proliferation response to WP. In an expanded analysis of two responders, the proliferative response to WP was mainly from CD4⁺ T cells with only a weak CD8⁺ T cell response
The distribution of CDI to WP was significantly different between patients (n=42) and controls (n=22); for patients the median (range) was 11.8 (1.0-323) and for controls it was 4.5 (0.8-12.8) (Fig. 1; p=0.0004; Mann-Whitney U-test).

The mean CDI was 32 ± 60 for patients and 4.9 ± 3.2 for controls. WP response was not correlated with duration of disease but there was a moderate inverse correlation with age (r=-0.35, p=0.025; data not shown). When a subgroup of 6 controls was matched with 19 patients for HLA-DR4, age and sex, the increased T cell proliferation in response to WP in the T1D group remained significant (p=0.038, Suppl Fig. S2).

**T cell responses to other dietary antigens moderately increased in type 1 diabetes patients:** Some patients displayed increased T cell responses to other dietary antigens including ovalbumin, an irrelevant dietary antigen, and to the celiac-related antigens (wheat gliadin, and α-gliadin 33-mer peptide) as well as the type 1 diabetes autoantigen, insulin (p=0.02, p=0.03 p=0.001, p=0.03 respectively) (Fig.2). We did not find significant differences between type 1 diabetes patients and control subjects in response to the recall antigen, tetanus toxoid, or the T cell mitogen, PHA (p=0.2, Fig.2). There was a positive correlation between WP T cell responses and T cell responses to OVA, gliadin and α-gliadin 33-mer peptide in type 1 diabetes patients (p=0.01, p=0.002 p=0.0001; data not shown). In contrast, we detected a weak, non-significant correlation between WP T cell response and insulin T cell response in type 1 diabetes patients (p=0.06; data not shown).

**High concentration of IFN-γ, IL-6 and IL-17A in supernatants of WP-stimulated PBMC:** Cytokines secreted by WP-stimulated PBMC were analyzed in the culture supernatant using flow cytometric bead arrays or ELISA (IL-17A) (Fig. 3). The concentration of the pro-inflammatory cytokines IFN-γ, TNF and IL-6 was higher in WP-stimulated PBMC from type 1 diabetes patients (p=0.03, p=0.008, p=0.001, respectively) whereas the counter inflammatory cytokines, IL-4 and IL-10 were not different. Removing one control from the IL-4 group analysis (circled symbol, Fig. 3) revealed significantly higher IL-4 concentration in the type 1 diabetes patient supernatants. However, the concentration of IL-4 was much lower than IFN-γ. The concentration of IL-17A in WP-stimulated PBMC culture supernatants from patients was increased compared with controls (Fig. 3, p=0.02).

**Association of HLA-DR4 risk alleles for type 1 diabetes with immunity to wheat polypeptides:** We analyzed HLA-DR and -DQ haplotypes in patients with type 1 diabetes and controls. The frequency of HLA haplotypes in the control group was similar to published reference populations [34] (data not shown). HLA-DRB1*03 and DRB1*04, which are associated with type 1 diabetes, were more frequent in type 1 diabetes patients compared with controls. Heterozygous DR3/DR4 individuals represented 29% of our type 1 diabetes patients but none of our controls was heterozygous for HLA-DR3/DR4 (data not shown). In addition, we compared the frequency of risk alleles in type 1 diabetes patients who were either responders or non-responders to WP (Table 2). Type 1 diabetes responders to WP carried HLA-DRB1*04 in 95% of cases, which was significantly higher than the non-responder group, (59%, p=0.01, Table 2). Distribution of T cell proliferation (CDI) to WP in type 1 diabetes patients with HLA-DRB1*03 or HLA-DRB1*04 or those heterozygous for HLA-DR B1*03/*04 is shown in Fig. 4. One of seven type 1 diabetes patients with HLA-DRB1*03/*04 showed positive responses to WP. In contrast, 10 of 20 type 1 diabetes patients who carried one risk allele HLA-DRB1*04 or 9 of 12 patients who carried
both risk genes, HLA-DRB1*03/*04 showed positive reactivity to WP.

The frequency of HLA-DQ2, the major celiac disease-associated allele, was not significantly different between type 1 diabetes patients and controls (p=0.6; data not shown). Importantly, the difference for HLA-DQ2 between responders and non-responders was not significant (p=0.75, Table 2). We also evaluated the HLA-DR and DQ haplotypes by high-resolution analysis in type 1 diabetes patients. A positive response to WP in patients was more frequently associated with the HLA-DR4 (DRB1*0401/4) DQB1*0301/2 haplotype and HLA-DR3/DQB1*0201 appeared to magnify the response (Fig. 4). Patients who were HLA-DQ3+ were also more likely to be responders (Table 2).

**T cell responses to WP inhibited by monoclonal anti-DR antibodies:**
To confirm the role of HLA-DR in the T cell response to WP, we evaluated the blocking effect of anti-HLA-DR mAb on PBMC of 14 type 1 diabetes patients. Monoclonal anti-DR antibodies blocked T cell responses to wheat polypeptides in type 1 diabetes patients whereas isotype control antibody did not prevent the T cell response (Fig. 5A). Anti-HLA-DR4 antibody had only a small inhibitory effect on T cell response to tetanus toxoid (Suppl Fig. S3). CBA cytokine analysis of the culture supernatants from WP-stimulated PBMC of type 1 diabetes patients showed that monoclonal anti-DR antibody significantly inhibited secretion of Th1 cytokines but not Th2 cytokines (Fig. 5B). WP-induced production of IL-17A was also blocked by the addition of anti-DR (Fig. 5B, p=0.008).

**DISCUSSION**

The foods we eat contain numerous non-self molecules which do not normally stimulate a pro-inflammatory immune response in healthy individuals [35]. In some diabetes-prone rodents [6,7,10] and humans [19-23] there is evidence that the gut mucosa is mildly inflamed and the epithelial barrier is leaky, providing a potential entry point for non-self antigens in the context of a pro-inflammatory cytokine imbalance that could promote autoimmunity [4,18]. The present results support this view. Although the origin of the antigens and immune cells that initiate or drive the β-cell-specific autoimmune response is not known, diet is an important factor influencing diabetes outcome, possibly by supplying a constant source of stimulatory antigens to the gut immune system [4].

There was moderately increased T cell proliferative response to other dietary antigens: OVA, gliadin and the celiac toxic gliadin 33-mer peptide (Fig. 2) but this was less pronounced than the response to WP. These data suggest a general impairment of oral tolerance in some type 1 diabetes patients [41], with the strongest and most frequent abnormal proliferative response being that induced by the mixture of wheat polypeptides.

We are aware of only one other study of WP response in patient PBMCs [29]. Klemetti et al. [29] showed an increased cell-mediated immune response to high concentrations of wheat gluten (400 µg/ml) in 24% of newly diagnosed type 1 diabetes patients. The proliferative responses in this study were low compared with the present study, possibly due to the high polypeptide concentration, different gluten fractions, culture conditions, proliferation assay and/or different genetic background of the subjects.

In keeping with reports that high concentrations of gliadin are cytotoxic [42], we found that 25 µg/ml of wheat protein extract inhibited response of PBMC whereas 6.2 µg/ml was optimum for our assay [29]. The CFSE assay used here not only permits identification of individual cell populations by flow cytometry but is also more sensitive than the thymidine assay [33]. Therefore, the present analysis permitted us to detect wheat
specific CD3+ T cell proliferation using a low, non-inhibitory dose of WP.

Additional evidence linking the development of diabetes in humans to wheat comes from epidemiological studies [27,28], and reports of immune responses of patient tissues to WP [29,30]. Anti-gliadin antibodies have been reported in newly diagnosed children with type 1 diabetes [43] and prospective studies of infants at high risk indicate that early exposure to cereals, particularly wheat, was linked to appearance of islet autoantibodies [27,28]. Furthermore, a significant subset of type 1 diabetes patients display celiac disease autoantibodies [44]. CD3+ lamina propria and intestinal epithelial lymphocytes (IEL) were increased in 20% of type 1 diabetes patients receiving rectally instilled gliadin [45]. Immunohistochemical evaluation and culture of jejunal biopsies from tissue transglutaminase antibody negative type 1 diabetes children with WP increased frequency of activated CD25+ cells in the lamina propria as well as expression of HLA-DR in the crypts in association with enhanced infiltration of the epithelium by CD3+ cells [30]. Therefore, the present results are consistent with those of Auricchio et al. [30] that a subset of type 1 diabetes patients displayed signs of inflamed gut mucosa and increased immune reactivity to wheat polypeptides.

Type 1 diabetes has traditionally been thought of as a T cell-mediated disease associated with high levels of the Th1 cytokine, IFN-γ. It now seems likely that type 1 diabetes is the result of dysregulation within a broader network of immune cell types [46]. For example, the role of the recently discovered Th17 cells and the extent to which there is a dysregulation of communication between Th1, Th2 and Th17 cells in human diabetes remains unclear. We observed WP-induced T cell proliferation in nearly half of our type 1 diabetes patients but not in healthy controls. This response was mixed in nature accompanied by increased production of pro-inflammatory cytokines IFN-γ, TNF, IL-6 and IL-17A as well as the counter-inflammatory Th2 cytokine, IL-4. The high concentrations of IFN-γ and IL-17A suggest that Th1 and Th17 cytokine-producing, WP-responsive CD3+ cells were activated in association with a pro-inflammatory condition in the gut [6,30]. IL-6 was present in WP culture supernatants at very high levels, 4-6 times those of IFN-γ. TNF was also increased but to a much lesser extent. IL-6 and TNF are pro-inflammatory cytokines that promote the secretion of IL-17 by Th17 cells and block the production of Foxp3+ Treg cells [47]. We did not observe a significant increase in IL-10 in response to WP, making it unlikely that Th17 originated from IL-17 regulatory cells that produce both IL-10 and IL-17. It seems unlikely that such high amounts of IL-17 could originate from CD8+ T cells because expansion of WP-responsive T cells was predominantly from CD4+ cells, and IL-17A production was blocked by anti-DR antibody.

High levels of IFN-γ were produced in response to WP whereas the concentration of the Th2 cytokine IL-4, although significantly increased, was only one third that of IFN-γ, suggesting a predominance of Th1 over Th2 cells. Although both of these cytokines can inhibit development of Th17 cells from naive precursors [47], it has been suggested that committed Th17 cells are not affected [46]. Therefore, we favor the interpretation that WP stimulates the production of IL-17A from previously committed Th17 cells. While IL-17A can be produced under certain circumstances by CD8+ T cells and members of the innate immune system, γδT cells and NKT-cells, Th17 cells are the major producers of IL-17A. Thus, the overall cytokine pattern observed in WP-stimulated PBMC from type 1 diabetes patients suggests a predominant Th1 and Th17 pro-inflammatory state consistent with the speculation that autoimmunity can occur.
when there is inappropriate cross-regulation between Th1 and Th17 cytokine networks [46,47].

The role of IL-6 in type 1 diabetes remains controversial [48]. In the present study, patient PBMC cultured with WP secreted large amounts of IL-6, the origin of which is presently unclear. Nonetheless, some points are worth noting: WP response was mainly from CD4+ and not CD8+ cells, most responders were HLA-DR4 positive and treatment with anti-DR did not block production of IL-6. These results suggest IL-6 originated from non-T cells. Others report that IL-6 gene expression was upregulated 3-fold in monocytes from adult onset type 1 diabetes subjects [49]. Furthermore, over-expression of IL-6 in pancreas caused islet inflammation and induced B-cell differentiation, which may be important in the development of autoimmune disease [48]. The high concentration of WP-induced IL-6 further supports our previous proposal of a potential mechanism by which wheat can promote the development of diabetes involving induction of pro-inflammatory cytokines such as IL-6, IFN-γ and TNF-α by wheat antigens [4,9,13,50].

The human leukocyte antigen genes are the major genetic determinant of type 1 diabetes. More than 90% of Caucasian type 1 diabetes patients carry either HLA-DR3 or HLA-DR4 haplotype and a synergistic effect on type 1 diabetes risk is observed in HLA-DR3/4 heterozygous individuals [51]. Patients with type 1 diabetes and celiac disease share some HLA-associated risk genes such as HLA-DQ2 (HLA-DQB1*02) and HLA-DQ8 (HLA-DQB1*0302). The frequency of HLA risk genes is different between the two diseases: 90-95% of celiac patients are HLA-DQ2 and 5-10% are HLA-DQ8 [52] whereas up to 50% of diabetes patients are HLA-DQ8/DQ2 heterozygotes [53]. In our type 1 diabetes population, increased immune response to wheat polypeptides was not explained by shared genetic risk for celiac disease. The frequency of HLA-DQB1*02 was not significantly different between type 1 diabetes patients and controls (p=0.6), and we did not detect any significant differences for HLA-DQB1*02 between WP responders and non-responders (P=0.75) (Table 2). We also evaluated the HLA-DR and -DQ haplotype by high resolution analysis in type 1 diabetes patients. A positive response to WP in patients was more frequently associated with the HLA-DR4 (DRB1*0401/4) DQB1*0301/2 haplotype and HLA-DR3/DQB1*0201 appeared to magnify the response (Table 2, Fig. 4).

A genetic basis for WP response in type 1 diabetes patients is suggested by the finding that T cell proliferation and inflammatory cytokine production were blocked by anti-DR antibodies, and responders were nearly all HLA-DR4+. Others have observed enhanced expression of HLA-DR in the intestinal mucosa of children with type 1 diabetes [20,30] and gluten-induced T cell proliferation was blocked by anti-DR antibodies in two patients in a previous study [29]. Importantly, WP-induced T cell proliferation was not explained by an over representation of the major celiac risk gene HLA-DQ2. HLA-DQ2 prevalence was not different between patients and controls or between WP responder and non-responder patient groups, nor was the T cell response attributable to the presence of subclinical celiac disease as all our patients with type 1 diabetes were negative for antibodies against the pathognomic celiac disease autoantigen, tissue transglutaminase. These findings are consistent with other reports that the gut inflammation observed in type 1 diabetes patients is different from that seen in patients with celiac disease [20,54] and not necessarily related to HLA-DQ2 or HLA-DQ8 [30].

Therefore, the tolerogenic function of the gut immune system with respect to wheat
T cell response to wheat polypeptides in type 1 diabetes

Polypeptides was compromised in a large subset of patients with type 1 diabetes in an HLA-DR-restricted, diabetes-specific manner. In summary, almost half of our type 1 diabetes patients displayed increased proliferation when PBMC were cultured \textit{in vitro} with non-toxic concentrations of WP. The cytokine pattern was mixed having characteristics of a predominant Th1 and Th17 response with a lesser contribution of the Th2 cytokine, IL-4. The WP proliferative response occurred mainly in HLA-DR4 individuals and was blocked with anti-DR antibodies but was not due to the major celiac gene, HLA-DQ2. This demonstrated the presence of a mixed pro-inflammatory Th1/Th17 response to dietary wheat polypeptides that appeared to be diabetes-specific.

ACKNOWLEDGEMENTS

This work was supported by the Juvenile Diabetes Research Foundation and Canadian Institutes of Health Research. We are indebted to the volunteers and the following physicians: The Ottawa Hospital: Dr. Rene Wong, Dr. Janine Malcolm; Children’s Hospital of Eastern Ontario: Dr. Sarah Lawrence. We thank Nancy Hampton of The Ottawa Hospital, for HLA typing and for helpful discussions. Dr. Chaitan Khosla, Stanford University, and Dr. Hubert Kolb, German Diabetes Center, Düsseldorf, Germany provided the $\alpha$-gliadin 33-mer. We thank Dr. Hubert Kolb and Dr. Nanette Schloot (German Diabetes Center, Düsseldorf, Germany), and Dr. William Cameron, the Ottawa Hospital Research Institute for reviewing the manuscript.
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Table 1 Description of Subjects: Description of groups of control subjects and patients with type one diabetes.

| Group            | Number | Sex ratio f/m | Age mean, (range) | Duration of diabetes mean ± SD |
|------------------|--------|---------------|-------------------|--------------------------------|
| Type 1 diabetes  | 42     | 28/14         | 26.7 (8-41)       | 11.2 ± 7                       |
| Healthy controls | 22     | 11/11         | 24.5 (18-32)      | n/a                            |

Table 2. HLA-DR and -DQ in type 1 diabetes responders and non-responders T cell proliferation (CDI) to WP was evaluated in T1D patients. Patients with a CDI value greater than mean + 3 SD of the control group (CDI>14.6) were classified as responders. Those below were classified as non-responders. The percentage of individuals with the indicated HLA haplotypes in these groups was calculated.

| Subjects (n) | DR3 | DR4 | DR3/DR4 | DQ2 | DQ3 |
|--------------|-----|-----|---------|-----|-----|
| Non-responder | 22  | 41% | 59%     | 14% | 41% | 64% |
| Responder    | 20  | 50% | 95%     | 45% | 50% | 95% |

(p=0.01) (p=0.04) (p=0.75) (p=0.02)
FIG. 1. Antigen-specific CD3⁺ T cell proliferation 1.2 × 10⁶ CFSE-labeled PBMC from patients with type 1 diabetes or healthy controls were cultured for 8 d in the absence or presence of different concentrations of wheat polypeptides (WP). On day 8, cells were stained with Cy-chrome conjugated anti-CD3 mAb. Cell Division Index (CDI) was calculated based on a fixed number of 5000 CD3⁺ CFSEbright cells using the formula CDI = Number of CD3⁺, CFSEdim cells with antigen / Number of CD3⁺, CFSEdim cells without antigen (medium). The horizontal line indicates the mean.

FIG. 2. T cell proliferation in response to wheat polypeptides and other antigens or mitogen 1.2 × 10⁶ CFSE-labeled PBMC from patients with T1D or healthy controls were cultured for 8 d in the absence or presence of WP, OVA, gliadin, 33-mer, insulin, TT or PHA (see materials & methods for details). On day 8, cells were stained with Cy-chrome conjugated anti-CD3 mAb. Cell division index was calculated. A CDI value greater than the control mean + 3 SD (CDI >14.6) was used to define a positive response to WP. P values indicate statistical difference compared with controls. The horizontal lines indicate the means.

FIG. 3. WP-stimulated cytokine secretion by PBMC at day 8 of culture Box and Whisker plots with individual values for control and diabetic subjects. IFN-γ, TNF, IL-6, IL-4 and IL-10 were measured by flow cytometric bead array. IL-17A was measured by ELISA. Each plot includes the mean (dashed line), median (solid line), distribution, and range. (Note: For IL-4, the difference between control and T1D is significant when the circled outlier value in the control group is removed, p=0.03)

FIG. 4. Graphic representation of the relationship between wheat-induced T cell response and high resolution HLA diabetes risk alleles
Box and Whisker plots with individual values for diabetic subjects. Each plot includes the mean (bold line), median (solid thin line), distribution, and range. (A) Distribution of T cell proliferation (CDI) to WP in type 1 diabetes patients with HLA-DRB1*03, HLA-DRB1*04 or those heterozygous for HLA-DRB1*03/*04. A CDI value greater than mean ± 3 SD of the control group (CDI>14.6, dashed line) was used to define a positive proliferation response. One of the type 1 diabetes patients with HLA-DRB1*03/*04 showed positive responses to WP (circle). Type 1 diabetes patients who carried one risk allele HLA-DRB1*04 (10 of 20) or patients who carried both risk genes, HLA-DRB1*03/*04 (9 of 12) showed positive reactivity to WP. High resolution results of HLA-DR are labeled beside each individual. (B) Distribution of T cell proliferation (CDI) to WP in type 1 diabetes patients with HLA-DQB1*02, HLA-DQB1*03 or those heterozygous for HLA-DQB1*02/*03. High resolution analyses for HLA-DQB1*02 and *03 are labeled beside each symbol; the vertical arrow beside 0201 means all individuals in the column carry DQB1*0201. Note: Three of the 42 T1D individuals are neither DR3 nor DR4, and therefore they are not included in this figure. One individual of seven with DR3/non-DR4 in the left panel is heterozygous for DQ*02/*03, and was therefore placed in the middle column in the right panel, (DQ*02*03). Differences in high-resolution haplotypes between responders and non-responders were not evaluated statistically because of the small sample size.

FIG. 5. Role of HLA-DR in the T1D T cell and cytokine response to wheat polypeptides
(A) The T cell response to wheat protein (6.2 µg/ml) was calculated as CDI in 14 patients and compared with cells cultured in the presence of anti-HLA-DR mAb (5 µg/mL) or with isotype
control antibody. (B) Cytokine profiles of supernatants from WP-stimulated PBMC of patients were evaluated in the absence and presence of monoclonal HLA-DR antibodies using Th1/Th2 cytometric bead arrays for IFN-γ, IL-4, TNF, IL-10 and IL-6 or an ELISA kit for IL-17A.
Figure 1

![Figure 1](image1)

Figure 2

![Figure 2](image2)
Figure 3

![Figure 3](image)

Figure 4

![Figure 4](image)
Figure 5

(A)

(B)