

Chapter 1. General Introduction, Research Aims and Hypotheses of the Study

Introduction

Type 1 diabetes is an autoimmune disease in which autoreactive T cells and autoantibodies attack the pancreatic β cells leading to progressive, irreversible destruction of pancreatic β cells that result in pancreatic β cells' dysfunction and lesions (reviewed by Atkinsons and Eisenbarth, 2002 and reviewed by Bach, 1994). Clinical manifestation of the disease is associated with loss of 60%-80% of pancreatic β cells, resulting in absolute insulin deficiency leading to hyperglycemia, spontaneous ketosis, diabteic ketoacidosis etc. (reviewed by Notkins and Lernmark, 2001 and reviewed by Pinero-Pilona and Raskin, 2001). Type 1 diabetes is often called insulin dependent diabetes mellitus (IDDM) due to the lifelong clinical need of exogenous insulin to sustain life (reviewed by Mordes et al., 2004). Considering early age of the onset of the disease, it is also called juvenile diabetes (reviewed by Notkins and Lernmark, 2001).

The disease incidence has continuously risen over the past few decades with an annual rate of increase in disease incidence of 3.4 % in Europe (EURODIAB ACE Study Group, 2000). Although a stable incidence of type 1 diabetes has been observed in United States over the past few decades (reviewed by Gale, 2002), one in every 400 to 500 American children and adolescents are affected by type 1 diabetes (National Diabetes Statistics, USA,

2005 -<http://diabetes.niddk.nih.gov/dm/pubs/statistics/index.htm>). Besides the morbidity and mortality associated with the disease, the lifetime cost of diabetes (type 1 and type 2 diabetes) has been estimated at over a billion US dollars (American Diabetes Association, 1998), thus imposing heavy social and financial burdens worldwide.

Despite intense research and technological advances, the main reason behind this huge failure associated with type 1 diabetes is poor understanding of disease etiopathogenesis. Although genetic makeup predisposes individuals to type 1 diabetes, environmental factors modify the disease pathogenesis, especially during the stage that precedes type 1 diabetes (reviewed by Couper, 2001). Cow's milk proteins and enteroviruses have been identified as possible environmental triggers of type 1 diabetes (reviewed by Couper, 2001). In addition, natural ingredient, non-purified diet has been reported to be associated with very high diabetes incidence (70%) in experimental animal model of IDDM (Hoorfar et al., 1993). A CBD, wheat flour diet is capable of inducing the highest disease incidence (60%), among diets containing single protein sources (Hoorfar et al., 1993). Further investigations about the prevention of disease development in non obese diabetes (NOD) mice, fed a wheat gluten free diet by Funda et al., (1999) confirmed wheat gluten as a dietary diabetogen. However, whether gliadin or glutenin proteins are instrumental for diabetogenic activity of wheat gluten remains obscure (reviewed by Scott, 1996).

Earlier, Coleman et al. (1990), showed that a chloroform methanol (CM) soluble fraction of the Old Guilford 96 diet (OG 96), a natural ingredient rodent

open formula mouse diet significantly increased type 1 diabetes incidence in NOD mice. In contrast, the residue after the CM extraction decreased the disease incidence. They also suggested that the lipid fraction of the CM soluble extract of the OG 96 diet possesses diabetogenic potential (Coleman et al., 1990). Based on Coleman et al. (1990) study, Shi, 2003 (M.S. Thesis), conducted a study that hypothesized that the CM soluble sphingolipid fraction of wheat gluten possess diabetogenic potential. However, they found that the CM soluble sphingolipid fraction did not trigger type 1 diabetes. They also suggested the possibility that there was another diabetogen in CM soluble extract of wheat gluten (Shi, 2003-M.S. Thesis, Virginia Tech). Meanwhile, Rocher et al.(1995), identified immunogens α and γ gliadins to be CM soluble wheat proteins (Rocher et al., 1995).

Thus, based on the results of Shi, 2003 (M.S. Thesis) and findings of Rocher et al., (1995) study, we designed a retrospective study to detect gliadins in the CM soluble sphingolipid enriched extract which was used in a feeding study conducted by Shi, 2003 (M.S. Thesis).

Recently two major components of immune system– T helper 1 (Th₁) and proinflammatory cytokines (reviewed by Rabinovitch, 1998) and the gut immunohistopathological changes (reviewed by Vaarala, 2002 and reviewed by Couper, 2001) are receiving increasing attention in the area of IDDM pathogenesis. However, they were not studied in association with IDDM induced by CM soluble components. Therefore in order to assess whether the gliadin specific sphingolipid enriched extract (GSLEE) is capable of causing type 1 diabetes and

the mechanism of type 1 diabetes development due to GSLEE, we prospectively assessed pancreatic and jejunal histopathology changes and sera interferon gamma (IFN- γ), tumor necrosis factor-alpha (TNF- α) cytokine concentration changes in BBdp rats, an animal model of type 1 diabetes.

In summary, we attempted to identify the gliadin specific constituent of CM soluble sphingolipid enriched extract as a diabetogen, and to elucidate the mechanism behind the pathogenesis of type 1 diabetes induction by the CM soluble GSLEE fraction of wheat gluten. The recognition of possible dietary diabetogens from CM soluble extract of CBD and its disease pathogenesis mechanism is of great importance in type 1 diabetes prevention and therapy.

Aims of the Study

Although CM soluble constituents of wheat gluten have been found to possess diabetogenic potential in type1 diabetes, it has remained a challenge to identify the individual constituents from the CM extract of wheat gluten responsible for disease pathogenesis. The results of the preliminary study conducted in our lab failed to show sphingolipids from the CM soluble extract as a trigger for diabetes. Therefore this study, a follow up study attempted to examine the role of the gliadin fraction (α -gliadin and γ -gliadins) of CM soluble sphingolipid enriched extract of wheat gluten as a possible diabetogen.

In the present thesis, we explored the novel hypothesis that the CM soluble gliadin specific sphingolipid enriched extract (GSLEE) of wheat gluten

triggers type 1 diabetes by changing the pancreatic and jejunal histopathology and sera Th₁ and proinflammatory cytokine patterns.

Research Aim

To determine if the chloroform methanol soluble gliadin specific sphingolipid enriched extract of wheat gluten induces type 1 diabetes in BBdp rats by changing pancreatic and jejunal histopathology and changing sera IFN- γ and TNF- α cytokine concentrations.

Specific Aims

Aim 1- Detection of gliadin proteins in the CM soluble extract of wheat gluten and the sphingolipid enriched extract by protein assay and sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE).

Aim 2- Histological examination and scoring of histopathological characteristics of pancreatic tissue specimens of BBdp rats from 5 different dietary treatment groups; one of the diet containing the gliadin specific sphingolipid enriched extract (GSLEE) and one diet devoid of it.

Aim 3- Histological examination and scoring of histopathological characteristics of jejunal tissue specimens of BBdp rats from 5 different dietary treatment groups.

Aim 4- Immunohistochemical staining and enumeration of CD4 and $\gamma\delta$ TCR positive cells in jejunal tissue specimens of BBdp rats from 5 different dietary treatment groups.

Aim 5- Quantification of IFN- γ and TNF- α concentrations in sera of BBdp rats from 5 different dietary treatment groups.

Research Hypotheses

The chloroform methanol soluble gliadins (α -gliadin and γ -gliadins) present in the sphingolipid enriched extract of wheat gluten induce type 1 diabetes by inducing pancreatic and jejunal histopathology changes and inducing changes in IFN- γ and TNF- α cytokines.

The following null hypotheses were tested in this study-

- 1) H₀: There are no gliadin proteins in the CM soluble extract of wheat gluten and the sphingolipid enriched extract.
- 2) H₀: There are no significant differences in the insulinitis score, content of lymphoid tissue aggregates in pancreata due to the presence or the absence of the CM soluble gliadin specific sphingolipid enriched extract of wheat gluten in the animal diets.
- 3) H₀: There are no significant differences in the jejunal lymphocytic infiltration score, mucosal thickness, epithelial erosion and jejunal villi height due to the presence or the absence of the CM soluble gliadin specific sphingolipid enriched extract of wheat gluten in the animal diets.
- 4) H₀: There are no significant differences in the numbers of jejunal CD4

and $\gamma\delta$ TCR positive cells in BBdp rats due to the presence or the absence of the CM soluble gliadin specific sphingolipid enriched extract of wheat gluten in the animal diets.

5) H_0 : There are no significant differences in concentrations of IFN- γ and TNF- α in BBdp rat sera due to the presence or the absence of the CM soluble gliadin specific sphingolipid enriched extract of wheat gluten in the animal diets.

Chapter 2. Review of Literature

I. Type 1 diabetes, an autoimmune disease.

Autoimmune diseases are characterized by activation of pathogenic, autoreactive T cells and autoantibodies provoking the anatomical (tissue or organ) lesions and consequently functional alteration (reviewed by Bach, 1994). For example, in multiple sclerosis, autoreactive T cells attack the myelin proteins in the brain and spinal cord whereas in graves disease autoreactive antibodies attack the autoantigen thyroid-stimulating hormone receptor and induce organ specific autoimmune disease (reviewed by Marrack et al., 2001). Autoreactive T cells and autoantibodies have shown to attack the autoantigen transglutaminase to cause the organ specific autoimmune celiac disease (reviewed by Marrack et al., 2001). In a systemic autoimmune disease, rheumatoid arthritis T cell and immunoglobulins participate in synovial joints destruction (Matsumoto et al., 1999).

Type 1 diabetes, a organ specific autoimmune disease is characterized by selective destruction of the insulin producing β cells of islet of Langerhans (reviewed Atkinson and Eisenbarth, 2001). Several experimental findings support the hypothesis that type 1 diabetes is an autoimmune disease including : a) islet infiltrate called insulinitis constitutes activated T cells (reviewed by Atkinson and Maclaren, 1994); b) successful adoptive transfer of the type 1 diabetes to healthy neonatal NOD mice by splenic T cells from diabetic NOD mice, however

considerable number of splenic cells were found to be necessary for the transfer of disease in the nondiabetic recipient (Bendelac et al., 1987); c) presence of auto antibodies in type 1 diabetes patients (reviewed by Atkinson and Eisenbarth, 2001); d) treatment of antibodies against the autoantigen (ant-GAD antibody) significantly reduces type 1 diabetes incidence, insulinitis and delays disease onset (Menard et al., 1999); e) immunosuppression by cyclosporin interferes with disease progress (Carel et al., 1996); f) inability to prevent disease recurrence even after the segmental pancreatic graft transplantation (Sibley et al., 1985); and g) association of type 1 diabetes with other autoimmune disease like celiac disease etc. (reviewed by Maki and Collin, 1997), all of these findings provide copious evidence that type 1 diabetes is an autoimmune disease.

Involvement of both humoral and cellular immunity has been proposed in the pathogenesis of type 1 diabetes (reviewed by Beyan, 2003); however, T cells (cellular immunity) have been suggested to initiate destruction of pancreatic β cells (reviewed by Tisch and McDevitt, 1996). There are several studies suggesting the fundamental role of cellular immunity in type 1 diabetes. Yang et al., (1997) have shown that B cell deficient mice develop insulinitis and type 1 diabetes (Yang et al., 1997). Furthermore, a X-linked agammaglobulinemia patient who was deficient in B cells in periphery and autoantibodies associated with type 1 diabetes, developed type 1 diabetes. Thus the findings of this case study implied that type 1 diabetes could be initiated and get progressed in the absence of B cell or autoantibodies (Martin et al., 2001). Antibodies to food antigens like cow's milk protein beta lacto globulin (BLG), α - casein and β - casein

have been observed in type 1 diabetes patients and in healthy people as well, possibly due to increased intestinal permeability (Sarugeri et al., 1999 and reviewed by Akerblom and Knip, 1998). Furthermore, B cell depleted, purified T cells are able to induce type 1 diabetes when transferred to a neonatal recipient (NOD mice) with suppressed B cells which suggests that B cell auto reactivity is secondary to IDDM pathogenesis (Bendelac et al., 1988). On the other hand, transfer of autoantibodies from a diabetes donor can not induce disease in the recipient (reviewed by Anderson and Bluestone, 2005), thus demonstrating the prominent role of cellular immunity in IDDM pathogenesis.

Type 1 diabetes, T cell mediated disease

The critical role of the T cell mediated immunity in type 1 diabetes has been well established from the studies conducted in two experimental animal models of type 1 diabetes, NOD mice and BBdp rats. Presence of both CD4+ and CD8+ T lymphocytes has been shown to be necessary for the adoptive transfer of type 1 diabetes (Miller et al., 1988 and Bendelac et al., 1987). Further, Bendelac et al., (1987) suggested that there was cooperation between these two subsets of T cells in the process (Bendelac et al., 1987). Moreover it is clear that islet-specific T cell clones isolated from the pancreas of NOD mice can adoptively transferred insulinitis to disease resistant I-E⁺ NOD mice model (Nakano et al., 1991). In addition, insulin-specific T cell clones have shown accelerated the disease and adoptively transferred the disease in NOD mice (Daniel et al., 1995). Furthermore, antibody against T cells i.e. anti-CD3 antibody

injection resulted in reduction of insulinitis and diabetes in NOD mice by reducing the percentage of CD4+ and CD8+ cells in the spleen (Hayward and Shreiber, 1989). Of interest, complete or incomplete thymectomy reduced the spontaneous diabetes development to 3% and 9% respectively in neonatal mice suggesting thymus-dependent, cell-mediated autoimmune destruction of pancreatic β cells (Like et al., 1982), and the additional findings that an intrathymic injection of islets renders tolerance and consequently prevents IDDM in the experimental model of type 1 diabetes, BB/Wor rats (Koevary and Blomberg, 1992). Islet tolerance may have been established due to the enhanced activity and role of thymic antigen presenting cells (APCs). In addition, intrathymic injection of islets may have prevented anti-islet splenic immunoreactivity in diabetes prone rats, thus preventing disease development (Koevary and Blomberg, 1992). Taken together, all these studies demonstrated that type 1 diabetes is a T cell mediated autoimmune disease.

Although Miller et al. (1988), proved that both CD4+ and CD8+ T cell types are necessary for the disease pathogenesis (Miller et al., 1988), there are still ambiguities about their individual roles in type 1 diabetes. However, the findings that NOD B2m^{null} mice devoid of β 2-microglobulin that lacked class I expression and CD8+ T cells in the periphery failed to develop the disease, suggests that CD8+ cells are essential for the disease development (Wicker et al., 1994). The fact that inflamed islet mainly consists of CD8+ cells (Atkinson and Maclaren, 1994) supports the primary role of CD8+ cells in the disease pathogenesis. On the other hand, T cell clone studies have clearly shown that CD4+ cells

themselves are capable of damaging pancreatic β cells in the absence of CD8+ cells (reviewed by Dilts et al., 1999). One study has shown the regulatory mechanism of CD4⁺CD25⁺ cells in autoimmune diabetes via a decrease in pathogenic CD4+ lymphocytes, which hampers the IFN- γ production, and lymphocytic infiltration in pancreatic tissues, providing additional evidence for an important role of CD4+ lymphocytes in disease pathogenesis (Sarween et al., 2004).

Antigen presenting cells in type 1 diabetes

Dendritic cells and macrophages, an antigen presenting cells were found to be the first cell types invading islets in type 1 diabetes development which suggests the cardinal role of these cell types in antigen presentation in type 1 diabetes (Voorbij et al., 1989 and Jansen et al., 1994). A defect in the expression of co-stimulatory molecule was observed in dendritic cells in association of type 1 diabetes. Lower expression of B7.1 and B7.2 were found on dendritic cells from type 1 diabetes patients when compared to the control. This expression resulted in impairment of antigen presenting function of dendritic cells and of CD28/CTLA-4/B7 co-stimulatory pathway essential for regulation of immune responses. Thus this impaired cascade was considered to be responsible for type 1 diabetes pathogenesis (Angelini et al., 2005). Similarly macrophages are required for the activation of T cells into pancreatic β cell cytotoxic CD8+ T cells. Indeed, Jun et al.(1999) have shown that T cells from the macrophage depleted 8.3 TCR- β transgenic NOD mice were unable to differentiate into CD8+ T cells and therefore

prevented type 1 diabetes development or adoptive transfer of the disease in NOD SCID mice. The mechanism behind this phenomenon was found to be due to significantly lower IL-12 expression and production, which resulted into decreasing Th₁ immune response (Jun et al., 1999). In addition to these two cell types B lymphocytes were also observed to function as APC for the presentation of autoantigen GAD in autoimmune diabetes in NOD mice (Serreze et al., 1998).

II. Epidemiology of type 1 diabetes.

Type 1 diabetes is one of the most severe, chronic childhood diseases. Although the disease manifests in children and adolescents, with the highest incidence among children of age group 10–14 years (Karvonen et al., 2000), young adults constitute one third (37%) of the total IDDM population (Laakso and Pyorala, 1985).

The worldwide pattern of IDDM (WHO-World Health Organizations DiaMond Incidence study project -Multinational Project for Childhood Diabetes) has shown greater than 350 fold variation in disease incidence. This project included children \leq 14 years from 100 populations. Data collected from 1990-1994 revealed the highest incidence rate, 36.8/100,000 per year, in Sardinia followed by 36.5/100,000 per year in Finland. On the other hand, the data showed the lowest incidence rate, 0.1/100000 per year incidence rate in Zunyi, China and Caracas, Venezuela, (Karvonen et al., 2000). Extraordinary continental variations were also found with the lowest incidence rates in the Asian continent, intermediate incidence rates in the African continent and highest

incidence rates in the North American and the European continents (Karvonen et al., 2000). Furthermore, low-intermediate incidence occurred in the South American population as compared to North American population, which suggests an ethnic variation in type 1 diabetes incidence (Karvonen et al., 2000). Noticeable within country variations have also been observed for a few nations (Karvonen et al., 2000).

A consistent and significant increase in the type 1 diabetes incidence has occurred over the past decades (Zhao et al., 1999). For instance, in Switzerland childhood diabetes has increased from 7.8/100,000 in 1991 to 10.6/100,000 in 1999, with an annual average increase of 5.1% when entire population of newly diagnosed IDDM children <15 years was studied (Schoenle et al., 2001). Similarly, a significant increase of 2.49% per year (95% CI 1.089–3.91) in IDDM incidence has been observed in southwest of England (Zhao et al., 1999), whereas a 3.0% increase per year (95% CI 2.6–3.3, $P < 0.0001$) was observed globally during 1960-1996 (Onkamo et al., 1999). A striking feature of this type of increase is that there is pronounced increase in disease morbidity in the low incidence populations as compared to the high incidence populations (Onkamo et al., 1999). The diabetes incidence rate was stable in Finland with 36/100,000 children per year during 1987-1993, but after that a steep increase was observed in 1996 with 45/100,000 children per year (Tuomilehto et al., 1999). If this trend continues, incidence of type 1 diabetes will exceed 50/100,000 per year in Finland by 2010 (Tuomilehto et al., 1999), with an overall worldwide incidence 40% higher in 2010 than in 1998 (Onkamo et al., 1999).

According to the Center for Disease Control and Prevention (CDC), IDDM accounted for 5%-10% of total diagnosed cases of diabetes in the United States (http://www.cdc.gov/diabetes/pubs/pdf/ndfs_2005.pdf), with nearly 1 in every 400 to 500 American children and adolescents being affected by type 1 diabetes, according to the reports from the National Diabetes Statistics, USA, 2005 (<http://diabetes.niddk.nih.gov/dm/pubs/statistics/index.htm>).

III. Etiological factor of type 1 diabetes.

Both genetic and environmental factors have been implicated as triggers of type 1 diabetes, furthermore type 1 diabetes is considered to be caused by interactions among genetic and environmental factors (reviewed by Akerblom and Knip, 1998) leading to an inter-influence of these etiological factors during type 1 diabetes development. For instance, genetic background of the host has greater influence on virus induced type 1 diabetes development (Yoon et al., 1978).

Genetic factors

Genetic background is thought to be an important etiological factor in disease development. The cumulative risk for type 1 diabetes development among first degree relatives of type 1 diabetes is 4.4% (95% CI 3.1–5.7) for siblings aged 20 years and 2.6% for parents by age 40 years with 3.6% (CI 2.7–4.6) for father and 1.7% (CI 1.0–2.4) in mothers (Steck et al., 2005). The highest

risk was observed when type 1 diabetes was diagnosed at a very young age (Steck et al., 2005). In addition, a follow up study conducted in non diabetic identical twins of recently diagnosed IDDM patients for 24 years revealed that no more than 36% of the twins of IDDM patients will ever become diabetic. Thus the results of this study suggested that the risk to develop IDDM due to genetic factor is less than half the lifetime risk (Olmos et al., 1988).

The human leukocyte antigen complex (HLA) genes involved in antigen presentation are believed to be a major contributor to the genetic susceptibility of type 1 diabetes (reviewed by Bennett and Todd, 1996). Two candidate gene regions are associated with IDDM. The class II gene in the major histocompatibility (MHC), HLA region on the short arm of chromosome 6p 21.31 (IDDM1) has the greater effect. The other one is called IDDM2, and is located in the insulin gene region (INS) on chromosome 11p 15.5 with lesser effect. These two genes account for 40%-60% of familial clustering in IDDM (reviewed by Bennett and Todd, 1996). Moreover, when 123 type 1 diabetes patients and their families were investigated for HLA-DR and MT1, MT2, MT3 genotypes, 98% of the type 1 patients were found to possess either DR3 (relative risk = 5.0) or DR4 (relative risk = 6.8) antigens or both antigens, in addition a significant shift toward DR haplotype as compared to entire HLA haplotype (A, B, C, DR) was observed in healthy and diabetic siblings as well (Wolf et al., 1983). Todd et al., (1987) have also shown the presence of either HLA-DR4 and / or HLA- DR3 in 95% of IDDM population (Todd et al., 1987).

Data from deoxy-ribose nucleic acid (DNA) sequencing analysis of 3 IDDM patients and several controls indicated that the HLA-DQ β alleles also help to determine the disease susceptibility or resistance (Todd et al., 1987). Heterozygosity of DQ α 1*03-DQ β 1*0302 (DQ8) allele with DQ α 1*05-DQ β 1*0201 (DQ2) allele (DQ α 1*03-DQ β 1*0302 (DQ8) / DQ α 1*05-DQ β 1*0201 (DQ2) genotype) confers the highest significant risk for type 1 diabetes. Also the rare haplotype DQ α 1*03-DQ β 1*02 was found to be associated with early onset of IDDM (van Autreve et al., 2004)

Moreover the amino acid (AA) residue occurring at position 57 of the HLA DQ β chain is linked to disease susceptibility or resistance and is proposed to decide disease fate. Two Asp (aspartic acid)-57 negative DQ β alleles promote full disease susceptibility. On the other hand, the presence of two aspartic acids (Asp)-57 DQ β alleles confers complete resistance (Todd et al., 1987). In addition, contribution of presence of AA at positions 52 (arginine) on DQ α -chain to type 1 diabetes susceptibility has been demonstrated (Gutierrez-Lopez et al., 1992). Moreover these two alleles (DQ β non- Asp 57 and DQ α Arg 52) synergistically increased the risk of type 1 diabetes development (Gutierrez-Lopez et al., 1992).

The possible role of the 15 putative IDDM susceptibility loci designated IDDM1-IDDM15 have been investigated by genome screening, but little or no supportive evidence was obtained for most of these genes as a risk factor, suggesting a very low risk for type 1 diabetes associated with these multiple loci (Concannon et al., 1998).

On the other hand, investigators of a cohort twin study reported the estimated disease concordance rate in identical twins was 36% over a follow up of 24 years, when 49 non-diabetic identical twins of recently diagnosed type 1 diabetes patient were investigated (Olmos et al., 1988). In another study by Barnett et al., (1981), ~ 54% of pairs in the insulin dependent diabetes (IDD) group were found concordant when total of 200 pairs of identical twins were studied (Barnett et al., 1981). The authors of both of these studies concluded that type 1 diabetes does not have an entirely genetic basis and non-genetic factors could operate over a finite or prolonged time period (Olmos et al., 1988 and Barnett et al., 1981) before the clinical onset of the disease.

Similarly, the result of a study conducted by Feltbower et al., (2002) in Bradford, UK suggested an increase in the rate of disease development in the offspring of a migrant South Asian population (average annual increase-6.5%) as compared to individuals in non South Asian populations (average annual increase-2.4%) over the period of 21 years. An important finding of this study was that at the beginning of the study in 1978, the incidence was very low in south Asian children, and then the incidence rate climbed up dramatically and finally overtook the incidence rate of the non-south Asian children. The result of this study could not be explained by genetic factors alone, implying some powerful environmental factor must have influenced type 1 diabetes etiology (Feltbower et al., 2002).

Thus the twin study data and migrant study data lead to the conclusion that the genetic component is essential, but not sufficient by itself in causing the

disease, and there is an important role of environmental factors in disease pathogenesis.

Environmental factors

Extensive epidemiological studies and studies in experimental animals over the years revealed that certain viruses and dietary components are two candidate risk factors among all the environmental factors causing type 1 diabetes, whereas genetic predisposition allows the disease to progress (reviewed by Akerblom and Knip, 1998).

Other environmental factors

Other environmental risk factors include toxins, nitrate/nitrite from drinking water and diet, climatological factors (reviewed by Akerblom and Knip, 1998) and pubertal years (reviewed by Dahlquist, 1998). These factors contribute to the disease pathogenesis by individual mechanisms. Dietary nitrate/nitrite if converted into N-nitroso compounds (nitrosamines and nitrosamides) serve as risk factors for type 1 diabetes (reviewed by Akerblom and Knip, 1998). N-nitroso compounds are capable of either damaging the β cells, probably by reducing the nicotinamide adenine dinucleotide (NAD) content of the cells (reviewed by Dahlquist, 1998), or triggering the immune responses against the β cells (MacFarlane and Scott, 2002). Toxins like streptozotocin (STZ) causes type 1 diabetes when administered at a very high single dose or when given in low multiple doses to individuals with genetic predisposition (reviewed by Scott and

Marliss, 1991). STZ induces type 1 diabetes directly through a mechanism of pancreatic β cell subtoxicity and apoptosis (reviewed by Scott and Marliss, 1991), and indirectly when apoptosized pancreatic β cell enhance the cytotoxic CD8+ T cell priming and autoantigen presentation to these cells (Zhang et al., 2002).

Climatological factors like lower temperature (mean monthly temperature) and fewer hours of sunlight are found to be associated with high disease prevalence in Sweden, pointing to the possible relation between vitamin D deficiency and the disease pathogenesis (Dahlquist and Mustonen, 1994). High IDDM incidence has been found to be associated with pubertal years, which is attributed to the growth rate and peripheral insulin resistance due to high levels of growth hormones (reviewed by Dahlquist, 1998). Recently stressful life events have emerged as etiological factor of type 1 diabetes as well (reviewed by Dahlquist, 1998).

Viruses

Rosenbauer et al. in 1999, from their epidemiological study, reported significant seasonal variation in the IDDM incidence, with highest disease incidence in summer and lowest disease incidence in spring (Rosenbauer et al., 1999). Diseases varying with season are often caused by viruses (reviewed by Jun and Yoon, 2001). To date, nine viruses have found to be associated with type 1 diabetes in animals and about six viruses in humans including common viruses Coxsackie virus B (CVB) and rubella (reviewed by Jun and Yoon, 2001).

Viral infection may occur during the intrauterine period (Hyoty et al., 1995), fetal and neonatal period when mothers have significantly increased levels of CVB-3 IgM antibodies implying maternal enteroviral infection (Dahlquist et al., 1995), or at early infancy (Sadeharju et al., 2003).

Several viruses- rotavirus, rubella, cytomegalovirus, Epstein-Barr virus, mumps and retrovirus infections have been considered to be associated with type 1 diabetes and a correlation between the enteroviral infection and type 1 diabetes has been studied and extensively reviewed (reviewed by Hyoty and Taylor, 2002). In utero and infancy, enterovirus CVB infections are found to induce β cell damage and clinical IDDM (Hyoty et al., 1995), whereas immunization with coxsackievirus B4-E2 delays the type 1 diabetes development in NOD mice (Davydova et al., 2003).

The ability of enterovirus Coxsackie virus B4-JVB (JVB-Coxsackie B virus prototype strains) to cause the disease was demonstrated when a Coxsackie virus B4 passaged 14 times in pancreatic β cell enriched culture which was then inoculated to SJL/J mice, type 1 diabetes was developed with all the clinical features in 80% of the inoculated animals. After staining the pancreatic β cells with fluorescein-labeled (FITC) antibody to Coxsackie virus B4, the presence of viral antigen was found (Yoon et al., 1978). Serological investigations by Hyoty et al., (1995) found higher frequency of IgM class enterovirus antibodies in patients having IDDM diagnosed at younger age (<3 years) when compared to the age matched controls indicating most recent CVB infection (Hyoty et al., 1995).

Several mechanisms for viral induction of autoimmune diabetes have been discussed which include : a) destruction of pancreatic β cells by rapid replication of viruses in β cells (reviewed by Jun and Yoon, 2001), b) activation and recruitment of macrophages to the islet and subsequent cascade of TNF- α , IL-1 β and nitrous oxide leading to the death of β cells (reviewed by Jun and Yoon, 2001), c) molecular mimicry, for example, the β cell autoantigen, tyrosine phosphatase (IA-2, IAR), shares a common sequence with capsid protein VP1 of enteroviruses. Antibodies induced by this conserved motif cross react with tyrosine phosphatase (IA-2, IAR) damaging pancreatic β cells (Harkonen et al., 2002), d) activation of pancreatic β cell specific effector T cells by viral infection (reviewed by Jun and Yoon, 2001) and e) activation of autoreactive T cells by sequestered antigens released by damaged pancreatic β cells (Horwitz et al., 2002).

However, a study by Foulis et al. (1997), ended with conflicting results. They developed the polymerase chain reaction (PCR) technique (for Epstein-Barr virus and Cytomegalovirus), the immunocytochemical technique (for Mumps) and in situ hybridization technique (for Epstein Barr virus and Coxsackievirus) to test for the presence of these common viruses in autopsied pancreatic tissues of IDDM patients. However, they failed to find the evidence of persistent viral infection for these common viruses in the diseased pancreatic tissues (Foulis et al., 1997). In addition, lymphocytic choriomeningitis virus (LCMV) and mouse hepatitis virus (MHV) have found to prevent type1 diabetes development in BBdp rats and NOD mice respectively (reviewed by Jun and

Yoon, 2001). Furthermore, the results of the prospective studies remain inconclusive due to an undefined viral nucleotide sequence responsible for the disease pathogenesis (reviewed by Hyoty and Taylor, 2002). Together these studies imply that viral infection may not be an etiological factor in type 1 diabetes.

Dietary factors

Diet has been shown to be a major determinant of the type 1 diabetes development in animal models of type 1 diabetes. Most of the dietary studies have been conducted in rodent models of type 1 diabetes, NOD mice and BBdp rats.

Diabetes in BBdp rats is etiopathologically similar to human IDDM. Diabetes susceptibility genes including three genes Iddm1, Iddm2 and Iddm3 are the risk loci in BBdp rats similar to the human IDDM (reviewed by Scott, 1996). In this model, the disease develops spontaneously and 50%-70% of both males and females develop type 1 diabetes by the age of 60-120 days in specific pathogen free conditions (reviewed by Scott and Marliss, 1991). Another important characteristic of the disease in BBdp rats is that the disease development is largely modified by the diet fed to them after the weanling period (reviewed by Scott and Marliss, 1991). Further, insulinitis in BBdp rat is morphologically similar to that observed in human IDDM (reviewed by Mordes et al., 2004).

In NOD mice, the diabetes develops spontaneously and the disease resembles IDDM in humans in terms of genetic predisposition, involvement of autoimmunity, presence of autoantibodies and presence of severe lymphocytic infiltration in the pancreatic islets (reviewed by Anderson and Bluestone, 2005). Although the disease is mostly observed in female NOD mice with a 60-80% disease incidence (diabetes onset at 12 to 14 weeks of age), males also develop the disease with a 20-30% disease incidence (reviewed by Anderson and Bluestone, 2005).

In both of these models, the cereal based diet (CBD) proved to be a strong diabetogen (reviewed by Scott, 1996 and Hoorfar et al., 1993), on the other hand, a marked delay in type 1 diabetes development was observed with a hydrolyzed casein diet (reviewed by Scott 1996 and Hoorfar et al., 1993). A casein hydrosylate was also found to prevent the disease development if fed to experimental animals during early life (Elliott et al., 1988).

Diabetogenic or diabetes protective effects of these diets have been thought to be due to different mechanisms. The most critical mechanism is due alteration of MHC class I expression on pancreatic β cells. Pancreatic β cell specific cytotoxic T cells' (CTL) clones are MHC class I restricted (Utsugi et al., 1996), because CTL can only react with cells presenting peptide associated with MHC class I molecules (reviewed by York and Rock, 1996). Diet is capable of modifying MHC class expression on the pancreatic β cells. Indeed, Li et al. (1995), observed that a plant based rodent diet, Purina 5001 diet, induced hyper-expression of the MHC class I molecules on the pancreatic β cells when

compared to a hydrolyzed casein diet induced MHC class I molecules expression on pancreatic β cells (Li et al., 1995), thus making β cells more susceptible to cytotoxic attack. Thus hyper expression of MHC class I molecules was found to be associated with insulinitis and diabetes incidence (Li et al., 1995).

Numerous studies have reported dietary protein as a potential risk factor for type 1 diabetes, whereas carbohydrate and fat fractions of the diet were found not to be associated with type 1 diabetes (reviewed by Scott, 1996). However, it was postulated that only intact proteins exhibit the diabetogenic activity. In one experiment, when the natural intact proteins of the diet were replaced by L-AAs, the incidence of diabetes was reduced to 15% from the normal incidence of 50%, thus suggesting that the presence of intact proteins in the diet is necessary for the development of the disease in genetically susceptible individual (Elliott and Martin, 1984).

Earlier, Elliott and Martin (1984) reported, a diabetes incidence of 52% in BBdp rats due to the addition of 1% skim milk powder to the semi-synthetic diet when compared to the 15% diabetes incidence due to semi-synthetic diet alone which suggests diabetogenic potential of cow's milk protein in BB rats (Elliott and Martin, 1984). Contrary to this, other investigators reported the highest diabetes incidence (93% in females and 57% in males) due to a milk-free standard diet. Further addition of BGG, (bovine IgG) a protein present in milk, to a milk-free diet accounted for lower diabetes incidence (67% in females and 0% in males) when compared to a milk-free diet. The authors concluded that cow's milk protein has a protective role in the type 1 diabetes development in NOD mice. The authors

further pointed out that the failure to exclude the milk proteins from diet prior of weanling and failure to conduct feeding study with cow's milk protein as a single variable may have been responsible for demonstrating cow's milk protein as a diabetes trigger in the earlier studies (Paxson et al., 1997).

A cereal based National Institute of Health (NIH) diet is the most diabetogenic (disease incidence 63.4%) in BBdp rats (reviewed by Scott, 1996). Since soybean meal and wheat gluten are the major components of the NIH diet, the diabetogenic potential of NIH diet was believed to be due to the protein component of wheat (75% proteins) and soybean meal (50 % proteins) (reviewed by Scott, 1996). Soybean meal and wheat flour diet have potential to cause type 1 diabetes and are associated with a higher disease incidence (45 % and 60% respectively) (Hoorfar et al., 1993). These investigators further suggested the possibility of wheat proteins, wheat gluten and wheat gliadin of wheat flour as diabetogens (Hoorfar et al., 1993).

Wheat proteins and type 1 diabetes

Wheat protein consists of gliadins, glutenins, albumins and globulins (reviewed by Veraverbeke and Delcour, 2002). A heterogeneous mixture of hydrated proteins, gliadins and glutenins form wheat gluten, an endosperm storage proteins (reviewed by Veraverbeke and Delcour, 2002). Many studies have attempted to test the diabetogenic activity of each of these proteins.

Wheat globulins and albumins are associated with low diabetes incidence (reviewed by Scott, 1996). More recently MacFarlane et al. (2003) identified the

WP5212 putative protein with AA sequence homology to wheat Gli1 (globulin 1) as a possible type 1 diabetes trigger. Significantly higher antibody activity to this WP5212 was detected in diabetic BB rats when compared to the control and asymptomatic rats. Further this antibody reactivity was found in correlation with islet damage in respective group's BBdp rats (MacFarlane et al., 2003). However the globulin protein's exact role in disease etiopathogenesis remains to be established.

The ability of a gluten free diet to delay type 1 diabetes incidence and to prevent the disease in most of the experimental animals suggested that wheat gluten possesses diabetogenic potential and is a major dietary risk factor for type 1 diabetes (Funda et al., 1999). In a separate study, wheat gluten protein was shown to be a diabetogen when fed to weanling BBdp rats up to 170 days of age among all the diets containing different protein sources (Scott et al., 1988). In addition, cellular immune responses to wheat gluten were observed in 24.1% of newly diagnosed type 1 diabetes vs 15.4% of long standing type 1 diabetes patients, suggesting that T cell reactivity to wheat gluten is associated with IDDM at the time of disease onset (reviewed by Akerblom and Knip, 1998). However the study conducted by Coleman et al. (1990), reported no increase in diabetes incidence due to supplementation of 10% wheat gluten to an American Institute of Nutrition (AIN)-76 diet (Coleman et al., 1990). But this may be due to the mice's exposure to this diet 4-5 weeks after the weaning period that might have missed the open-window period in which the diet can influence the development of type 1 diabetes (Coleman et al., 1990). Thus it is evident from these

experimental results that wheat gluten is capable of triggering and promoting type 1 diabetes in these two animal models of type 1 diabetes.

A number of investigators have attempted to explore the diabetogenic potential of glutenins and gliadins separately. For example, low molecular weight (LMW) glutenin obtained after treating wheat gluten with dilute acetic acid showed high diabetogenic activity as compared to the high molecular weight (HMW) glutenin, but the dilemma regarding the diabetogenic potential of the overlapping part between these two fractions remained unresolved due to a lack of a follow up study (reviewed by Scott, 1996). When the semi-synthetic diet with intact proteins was replaced by L-AAs (semi-synthetic amino acids) and supplemented with the 1% gliadin, a 35% disease incidence was observed as compared to 15% incidence due to L-AAs replacement for the original diet (Elliott and Martin, 1984). Thus gliadin was found to elevate the disease incidence, and feeding a diet containing all of AA from gliadin produced a disease incidence of 22% (reviewed by Scott, 1996). However, strikingly 0% disease incidence was observed when 1.5% gliadin protein was added to a hydrolyzed casein diet (reviewed by Scott, 1996). However this 0% result may be attributed to the hydrolysed casein's protective effect in IDDM, and to the very little amount of gliadins in the hydrolyzed casein diet. Unfortunately, to our knowledge, this is the only published study that described the effect of pure gliadin on the frequency of type 1 diabetes in clinical feeding trials.

IV. Chloroform methanol soluble fraction of wheat gluten and type 1 diabetes.

When looking at morbidity, mortality and heavy costs associated with type 1 diabetes, identification and confirmation of the new dietary diabetogen and dietary modulators is of utmost importance, particularly with regard to the possibilities for inexpensive preventive or intervention measures for the people at higher risk of IDDM with appropriate dietary information (reviewed by Scott and Marliss, 1991).

Treating diets with various solvents was another important step taken in this direction to detect the various dietary diabetogens. The New Zealand Rodent Diet was extracted into water and separated by the dialysis into two fractions, a LMW and HMW fraction. The LMW fraction, when added back to the Pregestimil diet, was found to increase type 1 diabetes incidence leading to the conclusion regarding the nondiabetic nature of water soluble HMW fraction and the water insoluble fraction of New Zealand Rodent Diet (reviewed by Scott and Marliss, 1991).

A similar approach was followed by Coleman et al. in 1990. They extracted OG96, a cereal based diet (CBD) in 2 liters of CM (2:1)/Kg of OG96 and evaporated the solvent. When the extract and the residue were added back into an AIN-76 diet, the resultant diets produced respective diabetes incidence of 75% and 30% as compared to the 17% and 59% of respective diabetes incidence due to AIN-76 and OG96 diets alone at the age of 30 weeks. Thus, the

CM extract of OG96 diet significantly increased the diabetes incidence as compared to the AIN-76 diet. They concluded that the CM soluble fraction of the natural ingredient diet is a diabetes trigger and may be lipoidal in nature and may not be protein (Coleman et al., 1990).

Shi W, 2003 M.S. Thesis, Virginia Polytechnic Institute and State University (to be published).

Shi, 2003 (M.S. Thesis), conducted an investigation based on the Coleman et al. (1990) study to determine the possible role of CM soluble lipid fraction of CBD in disease pathology. She extracted wheat gluten with three volumes of CM (2:1). The resulting residue was extracted a second time with three volumes of CM (2:1) mixture and the supernatants of these two extraction procedure were subjected to a Folch wash to remove non-lipid components and finally total lipids were obtained. The sphingolipid enriched extract was obtained by fractionation of total lipid with silica gel chromatography followed by saponification of the acetone fraction. This sphingolipid enriched extract was used in an animal feeding study to investigate if the CM soluble sphingolipid enriched fraction from wheat gluten is able to influence the incidence of type 1 diabetes. The BBdp rats were randomly assigned to one of the five dietary feeding groups with 20 rats/group and an animal feeding study was conducted. The dietary ingredients according to each type of diet used in the Shi, 2003 study (M.S. Thesis), are listed in Table 1. Table 2 shows the diabetes incidence in the Shi, 2003 (M.S. Thesis) study.

Table 1. Dietary ingredients (expressed in %) according to five different dietary treatments adapted from Shi, 2003 study (M.S. Thesis).

Ingredients	HC	NTP¹ 2000	WG²	WGSLF³	HCSL⁴
Corn Starch	53.00	-	49.00	48.10	53.00
Sucrose	12.00	-	12.00	12.00	12.00
Casein Hydrolysate	18.00	-	-	-	18.00
Wheat Gluten	-	-	23.40	-	-
Wheat Gluten (ICN)	-	-	-	23.60	-
Ground Wheat	-	22.26	-	-	-
Ground Corn	-	22.18	-	-	-
Wheat Middlings	-	15.00	-	-	-
Soy Oil	7.00	3.00	6.10	6.80	3.40
Corn Oil	-	3.00	-	-	-
Soy Oil Containing Wheat Sphingolipids (extracted from wheat glutens)	-	-	-	-	3.60
Cellulose-Type Fiber	5.00	-	3.92	3.92	5.00
Soybean Meal (49% proteins)	-	5.00	-	-	-
Fish Meal (60% proteins)	-	4.00	-	-	-
Dried Brewer's Yeast	-	1.00	-	-	-
Alfalfa Meal (17% proteins)	-	7.5	-	-	-
Oat Hulls	-	8.5	-	-	-
Purified Cellulose	-	5.5	-	-	-
AIN-93G Mineral Mix⁵	3.50	0.50	3.50	3.50	3.50
AIN-93G Vitamin Mix⁶	1.00	0.50	1.00	1.00	1.00
Choline Bitartrate	0.20	-	0.20	0.20	0.20
L-Cysteine	0.30	-	0.30	0.30	0.30
L-Lysine	-	-	0.50	0.50	-
L-Threonine	-	-	0.08	0.08	-
Sodium Chloride	-	0.30	-	-	-
Calcium Phosphate, Diabasic (USP)	-	0.40	-	-	-
Calcium Carbonate (USP)	-	0.90	-	-	-
Choline Chloride (0% choline)	-	0.26	-	-	-
Methionine	-	0.20	-	-	-
Total	100	100	100	100	100

Continued.....

- 1.- Diet contain approximately 8.2% fat; 14.6% proteins; 10.7Z% moisture; 9.9% crude fiber; 52% of carbohydrate.
- 2.- No tert-butylhydroquinone added, soy oil contains 200 ppm BHA, BHT. Diet is made to contain 18% of proteins.
(Proximate analysis- %crude fat : 0.9%; % crude proteins: 76.8%; % moisture: 6.2%; % ash : 0.9%; % carbohydrate : 15.1%).
- 3.- No tert-butylhydroquinone added, soy oil contains 200 ppm BHA, BHT. Diet is made to contain 18% of proteins.
(Proximate analysis- %crude fat : 0.2%; % crude proteins: 76.3%; % moisture: 6.6%; % ash : 1.05%; % carbohydrate : 15.1%).
To make the diet more palatable, pellets were coated with a sugar-based, artificial maple syrup-flavored liquid.
- 4.- For addition of sphingolipid containing soy oil, split the amount half ($946/2= 473$ ml soy oil) in the first two batches of diet.
- 5.- Supplies per kg/diet: calcium carbonate-357g; potassium phosphate monobasic -196g; potassium citrate-70.78 g; sodium chloride-74 g; potassium sulphate-46.6 g; magnesium oxide-24.3 g; ferric citrate-6.06g; zinc carbonate-1.65 g; magnesium carbonate-0.63 g; cupric carbonate-0.31g; potassium iodate-0.01g; sodium selenate-0.01025g; ammonium paramolybdate-0.00795 g; sodium-metasilicate-1.45 g; chromium potassium sulphate-0.275 g; lithium chloride-0.0174 g; boric acid-0.0815 g; sodium fluoride-0.0635 g; nickel carbonate, hydroxide, tetrahydrate-0.0318 g; ammonium vandate – 0.0066 g; sucrose-220.716 g.
- 6.- Supplied per kg/diet-nicotinic acid-3 g; calcium pentotenate-1.6 g; pyridoxine HCl-0.7g; thiamine HCl-0.6 g; riboflavin 0.6 g; folic acid 0.2g; D-biotin-0.02 g; vitamin B12 (0.1% mannitol)- 2.5 g; DL-a tocopheryl acetate (500IU/g)-15 g; vitamin A palmitate (500,000IU/g)- 0.8 g; vitamin d3 (cholecalciferol, 500,000IU/g)-0.2 g; vitamin K (phylloquinnone) -0.0075 g; sucrose-974.705 g.

Table 2. Effect of dietary treatments on the diabetes incidence (expressed in %), adapted from Shi, 2003 study (M.S. Thesis).

	A) HC (n=20)	B) NTP 2000 (n=19)	C) WG (n=19)	D) WGSLF (n=19)	E) HCSL (n=20)
70 days	5	10.5	26.3	15.8	20
80 days	10	45.4	42.1	31.6	25
90 days	20	68.4	47.4	52.6	30
100 days	25	68.4	52.6	57.9	40
110 days	30	73.7	52.6	57.9	40
125 days	30	78.9	57.9	57.9	40

1. HC-Hydrolyzed casein based diet, the negative control.
2. NTP 2000-National toxicology program 2000 diet, the positive control.
3. WG-Wheat gluten based diet.
4. WGSLF- Wheat gluten based with sphingolipid enriched extract removed from the diet.
5. HCSL-Hydrolyzed casein based diet with sphingolipid enriched extract added to it.
6. A, B, C, D, E -Abbreviations used for the respective dietary treatments.
7. Diabetes incidence is expressed in %.

Adding back the CM soluble sphingolipid enriched fraction to the HC diet resulted in significantly higher diabetes incidence as compared to HC diet at age 70 days, 80 days and 100 days in BBdp rats, whereas after that period, no significant difference in diabetes incidence was observed between the HC diet and the HCSL diet groups as shown in the Table 2.

Although the result of this study suggested that the CM soluble lipid fraction of the wheat gluten is a diabetes promoter at earlier stages. However, the overall results do not lead to the conclusion that CM soluble sphingolipid

enriched extract is type 1 diabetes trigger because after removing the sphingolipid enriched fraction from wheat gluten based diet (WG), the resulting diet, wheat gluten based with sphingolipid enriched extract removed from the diet (WGSLF) produced similar diabetes incidence as WG diet (Shi W, 2003 -M.S. Thesis, Virginia Tech).

At the same time, other studies failed to establish a link between type 1 diabetes and the type 1 diabetes triggerer /promoter activity of lipids. Hoorfar et al. (1993), defatted the soybean with organic solvent hexane and the resulting defatted residue did not prove to have a beneficial effect on the diabetes incidence (Hoorfar et al., 1993). An organic solvent hexane soluble extract of an NIH diet did not affect the diabetogenicity. The authors concluded that the hexane soluble lipid did not possess diabetogenic activity (reviewed by Scott, 1996).

Conversely, according to the published research, there is abundant evidence suggesting the immunosuppressive role of lipoidal components in type 1 diabetes. At the International Symposium held in Ottawa, Ont., Canada, Dr. E. Colle reported that a diet containing 15% fish oil accounted for significantly low diabetes incidence as compared to the chow. In the same symposium, Dr. V. E. Kelley discussed fish oil rich in n-3 polyunsaturated fatty acids (PUFAs) as a potent immunosuppressant (reviewed by Scott and Marliss, 1991). Furthermore, diet low in linoleic acid lowered T cell dependent immune responses in control and in streptozotocin diabetic rats, thus demonstrating the immune suppressor activity of the linoleic acid (reviewed by Scott and Marliss, 1991).

Immune suppressive activity of dietary fat was proposed to occur by a number of mechanisms. First, a fat enriched diet has the ability to modify the cytokine profile. Kleemann et al.(1998) observed that a fat enriched diet (fish oil) is able to increase the messenger ribonucleic acid (mRNA) expression of IL-10, a Th₂ cytokine and simultaneously suppressing the mRNA expression of IFN- γ , thus shifting the Th₁/Th₂ ratio to Th₂ in the gut. Whereas in pancreatic tissue, mRNA expression of IL-10 was found higher in BB rats fed fat enriched diet when compared to the control rats (Kleemann et al., 1998). Other studies proved that a n-3 PUFAs rich diet exerts a suppressive effect on the production of IFN- γ , splenic IFN- γ ribonucleic acid (RNA) expression (Fritsche et al., 1999) and synthesis of IL-1 and TNF- α (reviewed by Scott and Marliss, 1991). Dietary lipids also elevate IL-2 (lymphokine interleukin-2) secretion as was observed when mice were fed a low fat diet or one of the high fat diet rich in coconut oil, olive oil, safflower oil, menhaden oil. The IL-2 secretion level was found to be significantly higher in culture media of lymphocytes of mice fed an olive oil and safflower oil rich diet as compared to a low fat diet group's animals (Yaqoob and Calder, 1995), whereas IFN- γ secretion was found to be unaffected by the dietary lipid treatments. IL-2 signaling cascade is essential for the protective function of regulatory T cells, which prevent and protect against autoimmune diseases (Furtado et al., 2002). To elucidate suppressant mechanism of lipoidal compents, Dr M. T. Clandinin of the University of Alberta reported that the dietary intervention by polyunsaturated and saturated (P/S) fatty acid results into alteration of T and B cell membrane phospholipids components which are

possibly responsible for the tolerance induction and suppressor activity (reviewed by Scott and Marliss, 1991).

Given these recent findings and the results of the Shi, 2003 (M.S. Thesis) study, the lipid fraction of the CM soluble extract is unlikely to be diabetogenic in nature.

V. Chloroform methanol soluble gliadin constituents and their role in type 1 diabetes pathogenesis.

Gliadin proteins and their subtypes

Gliadins are an endospermic protein fraction of wheat gluten. These proteins belong to a prolamine family (reviewed by Shewry et al., 1995). Wheat gliadins are subdivided into four types according to their electrophoretic mobilities : α , β , γ , ω subtypes (Rumbo et al., 1999). α , β , γ gliadins are S-rich group proteins (intrachain disulfide bonds) whereas ω gliadins are S-poor group proteins (reviewed by Shewry et al., 1995).

Chloroform methanol solubility of wheat gliadins

Earlier studies conducted by Rocher et al.(1995), reported α -gliadins and γ -gliadins to be CM soluble. They extracted CM soluble protein from wheat endosperm flour. This extract was further fractionated by 0.05 M ammonium bicarbonate (Bi), and a Bi-pellet and Bi-supernatant were obtained. By using the reverse phase high performance liquid chromatography (RP-HPLC) of the Bi-

pellet and Bi-supernatants, further immunoblotting with celiac serum and finally microsequencing analysis, they found celiac immunoreactive α -gliadins and γ -gliadins as a subtype of gliadins, atypical gliadin N terminal proteins, three N terminal blocked proteins and a few unidentified LMW protein fractions. The α -gliadins consisted of 31 kilo dalton (kD), 35 kD, 38 kD and two of 45 kD fractions. The γ -gliadins consisted of one 40 kD of γ_2 -gliadin, 31 kD and 50kD of γ_3 - gliadin and two γ -gliadins 31 kD and 40 kD type with atypical gliadin N terminal proteins (Rocher et al., 1995).

Type 1 diabetes and celiac disease

The association between type 1 diabetes and celiac disease is well demonstrated in a number of ways. Both type 1 diabetes and celiac disease are autoimmune diseases associated with HLA DR3 and DQ2 (reviewed by Akerblom et al., 2002). Type 1 diabetes patients develop celiac disease with a prevalence of 1.8% - 2.6% (Sjoberg et al., 1998) as compared to the 0.1 (1 case /1000 people)-1 % (1 case /100 people) prevalence rate of celiac disease in the general population (reviewed by Maki and Collin, 1997). Also 9.3% of recently diagnosed type 1 diabetes patients had celiac disease associated antibodies in their sera profile. Whereas 6.6% of recently diagnosed type 1 diabetes patients had celiac disease and thyroid disease associated antibodies (Jaeger et al., 2001). Conversely, 11.1% of the celiac disease patients have at least one IDDM specific antibody in their serum (Ventura et al., 2000). These findings help to explain the coincidence of these two diseases in many individuals. Several lines

of evidence suggest the possibility of common antigens for both diseases. α -gliadins and γ -gliadins are already known as the antigenic toxic proteins in celiac disease (Friis et al., 1994). Thus these proteins might also act as an immunogenic trigger in type 1 diabetes pathogenesis.

Digestive fate of gliadin peptide and autoimmunity

Immunodominant gliadin peptides are resistant to pancreatic proteases and endoproteases processing. In addition, they are found to be resistant to intestinal surface exopeptidase DPPIV (dipeptidyl peptidase IV) and DCPI (dipeptidyl carboxypeptidase I) due to their rate limiting activity (Hausch et al., 2002). Thus gastro-pancreatic digestion and further intrinsic slow processing of gliadins by exopeptidase result in the production of substantially longer peptides *in vivo* (Hausch et al., 2002). In genetically susceptible individuals, these nondigestible proline rich gliadin peptides may accumulate in the intestine and gain access to the subepithelial lymphocytes by crossing intestinal barriers and thus may induce the intestinal mucosal immune responses via gliadin specific T lymphocytes (Hausch et al., 2002 and Jensen et al., 1995). In another mechanism, this fraction may gain entry into the peripheral system due to increased intestinal permeability and induce peripheral immune responses with the help of gliadin specific T cells (reviewed by Mowat and 2003 Jensen et al., 1995). Thus ultimately this unprocessed gliadin fraction serves as a trigger for autoimmune diseases.

Humoral and cellular responses to gliadins in type 1 diabetes

Dietary gliadins were considered to cause pancreatic β cell damage causing type 1 diabetes (reviewed by Dahlquist, 1998). Gliadin may also play a role in type 1 diabetes by eliciting B cell mediated humoral responses in genetically susceptible people. Anti-gliadin antibodies have been found in 9% of type 1 diabetes patients without known (diagnosed) celiac disease (Carlsson et al., 1999).

Gliadin elicits cellular responses as well. When 19 IDDM patients were exposed to 2 grams of a peptic tryptic digest of gliadin by rectal challenge, a significant increase in rectal lymphocytes i.e. (greater than mean + 1 SD) in lamina propria (LP) and epithelium $\gamma\delta$ + lymphocytes and of LP and epithelium CD3+ were observed in 20% of IDDM children (Troncone et al., 2003). In an organ culture study when small intestinal biopsies of type 1 diabetes patients (without serological markers of celiac disease) were challenged with gliadin, enhancement of CD54 and crypt HLA-DR were observed. In addition, intraepithelial CD3+ cells and CD25+ LP cells were significantly increased in gliadin exposed biopsies when compared to either control biopsies cultures with gliadins or type 1 diabetic biopsies cultured with media alone (Auricchio et al., 2004), thus suggesting the activation of cell-mediated gut mucosal immunity.

Together the last two studies suggest gliadin's potential to induce cellular immune responses in type 1 diabetes via the gut immune system.

VI. Gut immune system and type 1 diabetes.

Structure of the gut immune system

Due to its length and enormous surface area, the gut mucosal immune system forms the largest lymphoid organ housing most of the lymphocytes and immunoglobulins (Ig) (reviewed by Mayer, 2000). The unique feature of the gut immune system is that it encounters more antigens and it clearly discriminates between infectious organism and harmless antigens like food etc.(reviewed by Mowat, 2003). Other characteristic features of the gut immune system include: a) gut associated lymphoid tissue (GALT) which forms the major component of the gut mucosal immune system, b) most of the lymphocytes of gut immune system remain in an activated stage due to the continuous exposure to the largest antigenic load, c) gut associated lymphocytes express adhesion molecules $\alpha_4\beta_7$ and d) an abundance of IgA in gut mucosal secretions (vs IgG in serum) (reviewed by Mayer, 2000).

GALT is comprised of organized tissues and effector sites. Organized tissues like peyer's patches (PPs), mesenteric lymph nodes (MLNs) and smaller, isolated lymphoid follicles are responsible for the induction phase of immune responses (reviewed by Mowat, 2003). Mature PPs i.e. macroscopic lymphoid aggregates are primarily made up of large B-cell follicles and intervening T-cell areas (reviewed by Mowat, 2003). MLNs are gut-associated secondary lymphoid organs and they are responsible for the immune responses to antigens

encountered in the gut lumen. This is the site where presentation of soluble dietary antigens to naive T cells occurs, and this is the common place for peripheral and mucosal recirculation pathways. Lymphocytes scattered throughout the epithelium and LP of the mucosa comprises the effector sites of GALT (reviewed by Mowat, 2003).

Immune cells of the gut immune system

Lamina propria lymphocytes (LPLs)- IgA plasma cell types constitute the major part of LPLs along with T cells (CD4+ and CD8+ cells), B cells, macrophages and dendritic cells (DC) (reviewed by Mayer, 2000). Although 40% of LP cells are CD8+ T cells, they preferentially stay in epithelium (IELs). Similarly CD4+ T cells, although found in the LP, also reside in the villus-crypt unit (reviewed by Mowat, 2003). Integrin- $\alpha_4\beta_7$ is expressed by LPLs. Due to the presence of all these lymphocytes, physiological inflammation is the characteristic feature of the normal healthy LP (reviewed by Mayer, 2000).

Intestinal intrapeithelial lymphocytes (iIELs)- Almost all the iIELs population in the small intestine is CD8+, which prominently expresses $\gamma\delta$ T cell receptors (TCRs) in the murine model. iIEL $\gamma\delta$ T cell population has an immunoregulatory effect (reviewed by Hayday and Tigelaar, 2003). iIEL expresses selective $\alpha E\beta 7$ integrins, which have distinct functions in cell signaling rather in homing of lymphocytes (reviewed by Mayer, 2000).

Intestinal epithelial cells (IECs)-Intestinal epithelial cells are called nonprofessional APCs. They process and present antigen to T cells. The unique feature of IECs is that they express MHC class II molecules and also selectively activate suppressor CD8+T cell in rat and in human as well through non-classical CD1d molecules. This unique pathway may be responsible for the maintenance of oral tolerance and mucosal inflammation (reviewed by Mayer, 2000).

Similar to T cells belonging to LP, iLE, MLN compartment, intestinal DC cells belong to LP, PP and MLN compartments. They are crucial in antigen presentation, generation of regulatory T cells or IgA promoting T cells. Thus they are consequently crucial in oral tolerance induction and maintenance (reviewed by Mowat, 2003).

Mechanism of antigen encounter and gut immune responses

Usually the epithelial layer, epithelium and tight junctions protect the organisms and antigens from entering into the underlying lymphoid tissue (reviewed by Mayer, 2000). However, once the gut immune system encounters an antigen, intestinal microfold (M) cells pass on the mucosal antigen to the APCs in epithelium or the underlying dome region. Most of the DC subsets in PP act as APCs in the gut immune system. These APCs move to the B cell follicles and T cell region where they react with the naive lymphocytes. Transforming growth factor (TGF- β) and IL-10 and cellular signals delivered by the activated T

cells and DCs lead the B cells Ig to switch from IgM to IgA in PP. Then the primed lymphocytes from the PPs gain entry into MLN through draining lymphatics (reviewed by Mowat, 2003).

The lymphocytes are further differentiated within MLN and finally exit through the thoracic duct into the blood stream (reviewed by Mowat, 2003). Finally they reach and gain entry into the mucosa via receptor ligand interactions i.e. $\alpha_4\beta_7$ integrin - mucosal addressin cell adhesion molecule-1 (MAdCAM 1). MAdCAM 1 is highly expressed by mucosal vasculature (reviewed by Mayer, 2000). The priming of lymphocytes due to GALT antigens results in the upregulation of $\alpha_4\beta_7$ (reviewed by Mowat, 2003). Simultaneously the chemokine receptor 9 (CCR9) is also expressed by gut primed lymphocytes i.e. primarily by all CD4⁺ and CD8⁺ T cells in iLE and LP compartments of jejunum and ileum. The corresponding chemokine ligand CCL25/TECK (thymus expressed chemokine) is selectively expressed on small intestine epithelial cells and drives the T lymphocytes to that site (Kunkel et al., 2000).

Once lymphocytes enter the gut mucosa, maturation of B-cell into IgA-producing plasma cells and naive T lymphocytes into CD4⁺ and CD8⁺ cells take place. Most of these T lymphocytes reside in the LP; however, CD8⁺ T cells migrate preferentially to epithelium and CD4⁺ T cells redistribute in the villous-crypt. These LP CD4⁺ cells may acquire regulatory phenotype here, which maintain the oral tolerance (reviewed by Mowat, 2003). Thus trafficking is an important feature of the gut immune system. Immune cells leave the mucosa

through lymph capillaries, MLN, and emerge through the thoracic duct lymph, and recirculate to their final destination, i.e to their mucosal site of origin as well as other mucosal sites (reviewed by Mowat, 2003).

Central role of gut immune system in type 1 diabetes pathogenesis

There is convincing evidence to suggest that the gut immune system plays a critical role in the pancreatic β cell autoimmunity and disease pathogenesis. Increased GIT permeability usually precedes overt manifestation of disease and is probably responsible for the presentation of luminal antigens to the mucosal immune system (Meddings et al., 1999). GIT enteropathy (crypt hypertrophy and hyperplasia, villus shortening, increased numbers of iIEL, increase level of mucosal peroxidase activity and mucosal infiltration with CD4+ T lymphocytes) is consistently present in BBdp rats, and it precedes insulinitis (Graham et al., 2004). $\alpha_4\beta_7$ -integrin, a gut-specific homing receptor was found to be expressed on the GAD reactive lymphocytes and on islet cell antigen reactive lymphocytes associated with type 1 diabetes (reviewed by Vaarala, 2000). For instance, Paronen et al. (1997), successfully showed the selective higher expression of $\alpha_4\beta_7$ -integrin on GAD65-reactive peripheral blood lymphocytes and further depletion of PBMC with higher expression of $\alpha_4\beta_7$ -integrin, reduced the cellular responses of peripheral blood lymphocytes against GAD in 50% of IDDM patients (Paronen et al., 1997).

Parallel to enhanced $\alpha_4\beta_7$ -integrin expression, increased expression of mucosal addressin MAdCAM-I on the islet endothelium during the development

of diabetes in NOD mice have been reported (Hanninen et al., 1993) and finally disturbed gut immune function in type 1 diabetes has been extensively discussed (reviewed by Akerblom and Knip, 1998). Most importantly, the ability of environmental risk factors like diet and viruses/ bacterial immunostimulant components to modify the disease incidence by modifying lymphocyte functionality, cytokine expression and synthesis has been reported (Scott et al., 2002, Bellmann et al., 1997 and reviewed by Vaarala, 2000). All these observations suggest a strong link between the gut immune system activation and IDDM pathogenesis.

CM soluble gliadin and breakdown of oral tolerance in type 1 diabetes

CD4+CD25+ T regulatory cells characteristics and functions

CD4+CD25+ T cell are known as naturally occurring suppressor cells (reviewed by Shevach, 2002). CD25+ T cells constitute ~ 5-10% of peripheral CD4+ T cell population in naïve mice/ healthy human (Sakaguchi et al., 2001). Some of the important characteristics of CD4+CD25+ T cells are that they possess immunosuppressive activity i.e., they are capable to suppress both CD4+ T cells and CD8+ T cells. They are activated by a TCR mediated mechanism and they are anergic in nature. They do not produce IL-2 and they express accessory molecule CTLA-4 that is critical for their distinctive functions (reviewed by Sakaguchi et al., 2001).

CD4+CD25+ T regulatory cells and autoimmunity

Abnormality/deficiency associated with regulatory T cells is thought to be one reason for the development of autoimmune diseases in human (reviewed by Sakaguchi et al., 2001). The observation of reduced levels of CD4+CD25+ regulatory T cells in animal strains those are predisposed to autoimmunity further supports the association of deficiency of regulatory T cells and autoimmunity (reviewed by Murrack et al., 2001). A BALB/c splenic cell suspension devoid of CD4+CD25+ T cells when transferred to recipient resulted in autoimmune disease development. However, this disease development due to lack of regulatory T cell population could not be attributed to lymphocytopenia because these cells constitute just ~ 5-10% of T lymphocytic population. This finding suggests that although the normal immune system harbors self-reactive T cells, their activation are normally kept in control by CD4+CD25+ T cells (reviewed by Sakaguchi et al., 2001). On the other hand, treatment with CD4+CD25+regulatory T cells reverses autoimmune colitis (Mottet et al., 2003). These regulatory T cells mediate their suppressor activity by producing distinct cytokines. For example, in IBD these cell populations were found to produce IL-10 and TGF- β , which suppressed the inflammation (reviewed by Shevach, 2002).

In autoimmunity, these regulatory T cells migrate and recognize the organ specific antigen by chemokine-chemokine receptor path and thus they are retained in the inflamed organs or in the lymph nodes draining such organs (reviewed by Shevach, 2002). In intestine associated autoimmunity, it has been observed that these regulatory T cells home to MLN and the colon in intestinal

inflammatory diseases. After 10 weeks of transfer of regulatory T cells, the mean frequency of regulatory T cells increased significantly to 40.7% in MLN and 17.7% in LP. Once they are home to MLN and colon, they proliferate in MLN and LP compartments (Mottet et al., 2003). Thus regulatory T cells are important to development of autoimmunity.

Oral tolerance breakdown

IDDM is considered a manifestation of loss of immunologic tolerance i.e. an oral tolerance breakdown to antigens- i.e. mostly to dietary oral antigens, considering that there is a strong link between gut immune system and type 1 diabetes (reviewed by Vaarala, 2000).

The immune system's ability to discriminate self antigens from non-self antigens is called immunologic tolerance and enables the immune system to protect its own cells, tissues and from autoimmunity (reviewed by Buer et al., 2005). This tolerance consists of two types, central tolerance and peripheral tolerance. Central tolerance controls the induction of tolerance to self-antigens by clonal deletion of self reactive T cells in the thymus by the process of positive selection and negative selection and subsequently protecting T cell mediated immunity. In a similar way, self-tolerance induction of B-cells takes place in bone marrow. Peripheral tolerance controls tolerance to the peripheral antigens expressed or modified outside of the thymus or bone marrow by process of ignorance, anergy etc. (reviewed by Mackay, 2001 and reviewed by Buer et al., 2005).

Oral tolerance is the main compartment of peripheral tolerance in which lymphocytes in the peripheral lymphoid tissue are rendered nonfunctional or hyposensitive by prior administration of the same antigen (reviewed by Strobel and Mowat, 1998). The gut immune system, the major T cell organ, is the main component of oral tolerance because it provides tolerance to the food antigens and harmless microbes encountered in the gut (reviewed by Vaarala, 2000).

Oral tolerance is also mediated by regulatory T cells by the mechanism called active suppression (reviewed by Weiner, 1994). T cells with CD4⁺CD25⁺ phenotype have been observed to be generated in large numbers during peripheral tolerance induction by injection of antigen or during oral tolerance induction by administering the antigen orally. This cell type was not able to produce IL-2 *in vivo* similar to CD4⁺CD25⁺ T cells produced in thymus and they were also found to possess immunoregulatory properties. Thus they were considered parallel to the immunoregulatory CD25⁺CD4 T cells produced directly in the thymus in response to self-antigen exposure (Thorstenson and Khoruts, 2001).

Enhanced T cell responses have been observed in newly diagnosed type 1 diabetes patients to cow's milk protein BLG (Vaarala et al., 1996) and to wheat gluten (Klemetti et al., 1998), which indicates the development of poor tolerance to these proteins during early life. On the other hand, antigen administration of the diabetes promoting food in the early days of life (4-7 days) has been found to delay disease onset and prevented the disease in one third of the BB rat population (Scott et al., 2002). These two studies suggest that oral tolerance is associated with type 1 diabetes.

Oral administration of porcine insulin to NOD mice starting from the age of 5 weeks to 1 year delayed type 1 diabetes onset and reduced overall disease incidence due to oral tolerance induction via active cellular suppression by insulin, which is an autoantigen (Zhang et al., 1991). In another study conducted by Bergerot et al. (1999) oral treatment of insulin was shown to induce insulin β chain reactive regulatory CD4 T cells. This cell population further prevented adoptive transfer of type 1 diabetes in NOD SCID recipient. These regulatory cells were found to be capable of blocking the function and migration of diabetogenic effector T cells to the pancreatic islets observed in co-transfer experiments. These regulatory T cell's protective/suppressive effect was considered due to the IL-4, IL-10 and TGF- β cytokines secreted by them, which inactivate the effector cells. Indeed, the author observed that in presence of regulatory T cells, effector T cells lost their functionality by secreting fewer amounts of IFN- γ and IL-2 secrete significantly lower amount of IL-2, a key player in T cell proliferation and function. This low amount of IL-2 secretion results into loss of functionality of effector T cells and associated down regulation of IFN- γ production. Thus these findings suggest a critical role of this cell type in oral tolerance induction for insulin antigen (Bergerot et al., 1999).

The breakdown of oral tolerance may be the consequence of elimination/reduction of CD4+CD25+ regulatory T cell population (reviewed by Sakaguchi et al., 2001), poor gut maturation, gut permeability, altered cytokine pattern, altered lymphocyte activity, alteration in the colonization of micro flora

and the genetic background of the individual (reviewed by Wasmuth and Kolb, 2000).

Oral tolerance development is also dependent upon normal maturation of the gut mucosal tissue and its immunologic homeostasis (reviewed by Wasmuth and Kolb, 2000). Indeed, breastfeeding and longer duration of breastfeeding exert a protective effect on type 1 diabetes development via their effect on gut maturation (Mayer et al., 1988 and reviewed by Wasmuth and Kolb, 2000). Breast milk contains the growth factors- epidermal growth factor (EGF), TGF- α HGF (Hepatocyte growth factor), IGF-I (Insulin growth factor), IGF- II, TGF- β and erythropoietin (Epo) that are responsible for postnatal intestinal growth and maturation (reviewed by Cummins and Thompson, 2002). A two to three fold increase in prolactin induced tissue alkaline phosphatase and maltase activity, an indicator of growth, vitality and maturation of gut has been observed when compared to the EGF induced hydrolases activity (Bujanover et al., 2002).

The presence of gram-negative bacteria in the gut is found to play a key role in development of oral tolerance as well (reviewed by Wasmuth and Kolb, 2000). In mice, intestinal flora, especially segmented filamentous bacteria (SFB) and some strains of chlostridia together are necessary for the development of the gut immune system including expansion of iIELs, IgA producing cells and MHC expression. The iIELs, IgA producing cells and MHC-II expression in small intestine were found to be less evident in a germ free group of mice as compared to a SFB, Chlostridia, SFB+ Chlostridia group (Umesaki et al., 1999). In addition,

a Th₁ cytokine bias also contributes to disturbed oral tolerance mechanism (reviewed by Wasmuth and Kolb, 2000).

It is possible that in our study, gliadin specific sphingolipid enriched extract may have caused disturbance in regulatory T cells resulting in breakdown of oral tolerance and consequently resulted in disease pathogenesis. Recently disturbance of immune regulatory cells has been pointed as the important factor in Crohn's disease (reviewed by Seibold, 2005). In addition, the gliadin specific sphingolipid enriched extract may have altered the cytokines environment to Th₁ type and enhanced T cell immune responses because of presence of gliadins in gliadin specific sphingolipid enriched extract. There is strong possibility that these Th₁ biased cytokine profile and T cell responses might have affected the gut maturation and immunological homeostasis leading to disturbed oral tolerance development. The germ free environment to which the BBdp rats were exposed in their early infancy might have negatively affected the gut maturation process, helping lead to disturbed oral tolerance.

CM soluble gliadins and molecular mimicry mechanism in type 1 diabetes

Considering the association of molecular mimicry and autoimmune diseases (reviewed by Oldstone, 1998), there is a possibility that gliadins from the sphingolipid enriched extract induce type 1 diabetes by molecular mimicry mechanism. Molecular mimicry is the structural homology shared by two different molecules or their protein products (specific sequence) with dissimilar genes (reviewed by Oldstone, 1998). In a molecular mimicry mechanism, if the protein

fragment of a foreign invader (food /infectious agent) closely resembles a self protein fragment, the resulting activated immune cells (antibodies/ lymphocytes) will not only attack the foreign invader but also the self protein having a similar AA sequence (reviewed by Oldstone, 1998).

Out of all the autoantigens detected to date, GAD65 and IA-2 are the major autoantigens recognized by by their respective autoantibodies in IDDM (reviewed by Leslie et al., 1999). Recently Honeyman et al. (1998), reported that two IA-2 epitope peptides are 82% similar and 64% identical over 10-11 AA sequences in wheat protein (Honeyman et al., 1998). It is possible that 11 AA sequences may belong to gliadin and thus immune cells activated by this 10-11 AA gliadin peptide might have attacked the IA-2 membrane protein of β cells and consequently damaged pancreatic β cell, inducing type 1 diabetes.

VII. T lymphocytes of gut immune system and gliadin specific sphingolipid enriched extract induced type 1 diabetes.

Intestinal T lymphocytes and their cytokines are crucial for maintaining healthy mucosa, through the epithelial proliferation factor-KGF (Keratinocyte growth factor), and the destructive factor-MMPs (matrix metalloproteinases) induction (reviewed by MacDonald et al., 1999).

Gut associated lymphocytes are found to be the most diabetogenic ones. Jaakkola et al.(2003), isolated lymphocytes from the spleen, pancreas draining, gut associated and subcutaneous lymph nodes of young NOD mice. They then

transferred these lymphocytes into NOD SCID/SCID recipients devoid of endogenous islet specific immune responses. In donors, aged 3 weeks, only gut lymph node associated lymphocytes possess the diabetogenic potential (effector cells). Whereas in donors, aged 6 weeks, pancreas draining lymph node associated lymphocytes were most diabetogenic and gut lymph node associated lymphocytes spleen associated lymphocytes were found to have the intermediates were found to have the diabetogenic potential. The authors of this study suggested gut associated lymph nodes and GALT are the site for initial priming of the diabetogenic lymphocytes and pancreas draining lymph nodes are the amplification site. They also demonstrated the migration of GALT specific diabetogenic lymphocytes into islets (Jaakkola et al., 2003).

Additionally, it has been shown by Hanninen et al. (1996), that at early stages of the disease, the islet is highly infiltrated with lymphocytes with a mucosal phenotype ($\alpha_4 \beta_7$ -integrin^{high} and L-selectin^{low}). At the age of 8 weeks, they found 66 % of α_4^+ , 48 % of β_7^+ and 5% of L-selectin + lymphocytes, whereas at the age of 12 weeks 73 % of α_4^+ , 64 of % β_7 and 6% of L-selectin + lymphocytes were found. In contrast, at the advanced stage only 26% of the infiltrated lymphocytes had mucosal phenotype $\alpha_4\beta_7^+$ (Hanninen et al., 1996), thus demonstrating gut specific lymphocytes as key players in type 1 diabetes pathogenesis, especially at the early stage of disease development.

Intestinal CD4+ cells, characteristics and functions

Intestinal CD4+ T cells stay mostly in the LP, and they are also distributed in the villus-crypt unit (reviewed by Mowat, 2003). LP CD4+ T cells are derived

from the T lymphocytes from PP after encountering luminal antigens (reviewed by MacDonald et al., 1999). After uptake of antigens, recognition by CD4⁺ T cells take place in the intestine, the antigen-responsive CD4⁺ T cells acquire expression of the $\alpha_4\beta_7$ -integrin and the chemokine receptor CCR9 and then they leave the MLNs in the efferent lymph and after entering the bloodstream through the thoracic duct, exit into the mucosa through vessels in the LP (reviewed by Mowat, 2003). These LP CD4⁺ T cells may acquire the regulatory phenotype here which is critical for induction and maintenance of oral tolerance (reviewed by Mowat, 2003). These LP CD4⁺T cells exclusively express CD45R0, CD69, $\alpha_4\beta_7$, Fas, low levels of L-selectin, HLA-DR and CD25 and a minority are FasL⁺(reviewed by MacDonald et al., 1999). LP CD4⁺ cells function as local immune regulatory cells, they produce large amount of IFN- γ and also secrete IL-4 and IL-10. Usually LP CD4⁺ cells are unresponsive to the TCR mediated proliferation signals (reviewed by Mowat, 2003).

Intestinal CD4⁺T cells and type 1 diabetes

A marked increase in LP CD4⁺ T cells in BBdp rat have been demonstrated in type 1 diabetes, and it has been suggested that intestinal CD4⁺ T cells play a decisive pathological role in enteropathy in type 1 diabetes (Graham et al., 2004), because of their ability to recognize antigens presented by HLA-DQ2 or -DQ8s molecules (reviewed by Sollid and Jabri, 2005). These HLA-DQ2 or -DQ8s is found to be essential in type 1 diabetes as in celiac disease (reviewed by Sollid and Jabri, 2005).

There is a growing body of evidence that diet influences small intestinal pathogenic CD4⁺ cell depots in type 1 diabetes. The results obtained from a study by Chakir et al. (2005), showed a three times higher frequency of CD3⁺CD4⁺IFN- γ ⁺ cells in the MLN of NTP-2000 fed BBdp rats as compared to the frequency of CD3⁺CD4⁺IFN- γ ⁺ cells in the MLN from BB control (BBc) rats. However, in the rats fed with hydrolyzed casein diet, the CD3⁺CD4⁺IFN- γ ⁺ cells in the MLN were present at control levels (Chakir et al., 2005). In addition, *in vitro* exposure of CD4⁺ MLN cells from BBdp to wheat gluten resulted into significantly high cell proliferation demonstrating that CD4⁺T cells become activated by wheat gluten antigens. The author concluded Th₁ bias and intestinal CD4⁺ Th₁ cells have a critical role in IDDM (Chakir et al., 2005). Moreover, Troncone and colleagues (1998), reported mainly IFN- γ specific CD4⁺T cell clones activation subsequent significantly high IFN- γ production by gliadin in celiac disease (Troncone et al., 1998). Thus gliadins may possess the potential to activate IFN- γ specific intestinal CD4⁺ cells in type 1 diabetes, an autoimmune disease associated with celiac disease.

Intraepithelial $\gamma\delta$ T cells, characteristics and function

$\gamma\delta$ T cells constitute the major T cell population in the murine intestine, accounting for ~ 37 % of the total iIEL population when compared to peripheral lymphoid tissue where they contribute just ~4% (Deusch et al., 1991). The important feature of these iIELs is that they are exclusively CD4⁻CD8⁺ T cells with a TCR-associated CD3 complex (Goodman and Lefrancois, 1988 and Deusch et

al., 1991). $\gamma\delta$ T cells in the small intestinal epithelium have a phenotype distinct from the $\gamma\delta$ T cells in peripheral lymphoid tissues. They are CD3+ TCR α/β - and are CD2-, CD5-, CD4- CD8 β - and have a homodimer form of CD8 α only. On the other hand, 90% $\gamma\delta$ T cells in peripheral lymphoid tissues express the CD8 $\alpha\beta$ heterodimer, CD2+, CD5+ and CD4- (Kuhnlein et al., 1994).

It has been considered that intestinal $\gamma\delta$ TCRs recognize antigen in a non-classical MHC restricted manner i.e recognizes MHC class 1b molecules exclusively expressed by IEC (Kagnoff et al., 1998). iIEL $\gamma\delta$ T cells are known to be a crucial functional component of the gut mucosal immune system. This cell population was found to exert protective effects on intestinal inflammatory disease i.e in inflammatory bowel disease (IBD) by TGF- β production (Inagaki-Ohara et al., 2004). Thus these cells constitute the first line of defense at the mucosal level and link innate and adaptive immunity (reviewed by Beyan et al., 2003).

Immune homeostasis is also maintained by $\gamma\delta$ +T cells because signal from this cell population has been proven to have control over the development and activation of α/β T cells and $\gamma\delta$ +T cells *in vivo* (Kaufmann et al., 1993 and reviewed by Hayday, 2000). This control may be maintained through their lymphokine secretion, through a cell surface ligand network etc (Kaufmann et al., 1993). $\gamma\delta$ T cells also play a critically important role in the priming of the CD8+ T cells against parasite infection (Moretto et al., 2001), and in bacterial infection (Nomura et al., 1998). In addition, these cells exhibit cytolytic activity (reviewed by Hayday, 2000). Functional interaction between iIEL and environmental

antigens largely decides the lytic nature of these $\gamma\delta$ T cells because $\gamma\delta$ T iIELs observed in mice reared in standard condition exhibit lytic activity whereas mice reared in germ free conditions were without lytic activity (Lefrancois and Goodman, 1989).

Importantly, expression of epithelial cell KGF by activated iIEL cells expressing $\gamma\delta$ TCRs (not $\alpha\beta$ TCRs) suggests a major role for $\gamma\delta$ iIELs' in repair of injured epithelial cells to maintain epithelial integrity (Boismenu and Havran, 1994).

Although the presence of bacterial and parasitic agents is the normal requirement for the activation of $\gamma\delta$ T cells, an autoimmune process induced by activation of $\gamma\delta$ T cells has also been reported in testicular cell-induced autoimmune orchitis, which was believed to be due to a inflammatory signal or self derived antigens (Mukasa et al., 1999).

Intraepithelial $\gamma\delta$ T cells and type 1 diabetes

There is promising evidence that $\gamma\delta$ T TCR+ /CD8+ T cells play a critical role in the prevention and pathogenesis of autoimmune diabetes. In the adoptive transfer model, diabetes incidence was suppressed by $\geq 75\%$ after co injection of splenocytes from aerosol insulin treated mice with diabetic splenocytes. This was due to induction of CD8 $\gamma\delta$ T regulatory cells during mucosal tolerance induced by aerosol insulin in NOD mice. Even small populations of $\gamma\delta$ T cells from CD8 cells were found to be capable of preventing the disease. Whereas CD8 cell populations from insulin aerosol treated mice were not able to suppress the

disease when they were depleted in $\gamma\delta$ T cells, which strongly suggests that $\gamma\delta$ T cells have an immunoregulatory function in autoimmune diabetes (Harrison et al., 1996).

Significant decrease in peripheral $\gamma\delta$ T+ cells were observed in prediabetics as compared to the recently diagnosed type 1 diabetes patients, suggesting the important role of $\gamma\delta$ T+ cell population in the process of disease development (Kretowski, 1999).

Immunohistochemical analysis showed that the intraepithelial compartment and LP compartments have increased number of $\gamma\delta$ + T cells in type 1 diabetes pediatric patients as compared to individuals in healthy age matched control group (Westerholm-Ormio et al., 2003). Gliadin specific mucosal immune response and significant increases in LP and epithelium $\gamma\delta$ + lymphocytes have been observed in a type 1 diabetes patient (Troncone et al., 2003).

It is possible that gliadin antigens from the sphingolipid enriched extract were presented to $\gamma\delta$ T cells which might have proliferated and recruited themselves, increasing their number in the gut mucosa and possibly priming cytotoxic CD8+ cells that destroy pancreatic β cells and cause the autoimmune disease.

VIII. Mechanism of migration of gut associated lymphocytes to pancreatic islets to induce type 1 diabetes.

Lymphocyte infiltration is a pre-requisite for the pathogenesis of autoimmune disease, which causes tissue injury and tissue dysfunction in type 1 diabetes. This tissue specific migration of the autoreactive and diabetogenic lymphocytes to the pancreas is facilitated by various protein molecules-integrins, addressins (Yang et al., 1994 and reviewed by Norman and Hickey, 2005), chemokines and chemokine receptors (reviewed by Rottman, 1999).

$\alpha_4\beta_7$ -MAdCAM-1 pathways

The $\alpha_4\beta_7$ -MAdCAM-1 pathway for lymphocyte trafficking to mucosal sites consists of $\alpha_4\beta_7$ -integrins as a receptor and MAdCAM-1 as a ligand (reviewed by Carlos and Harlan, 1994). $\alpha_4\beta_7$ -integrins are also called mucosal homing receptors. These integrins are exclusively expressed on gut specific lymphocytes leading to their homing to mucosal sites i.e. Peyer's patches and intestine (Hamann et al., 1994). On the other hand, MAdCAM-1 is selectively expressed on high endothelial venules (HEV) of mucosal lymphoid organs like PPs (reviewed by Carlos and Harlan, 1994) and in the vessel of the pancreatic islets (Yang et al., 1994). They also direct lymphocytes to lymphoid organs and other mucosal sites (reviewed by Carlos and Harlan, 1994 and Hamann et al., 1994).

Hanninen et al. (1993), investigated the role of adhesion molecules in the migration of T lymphocytes to the pancreatic tissue by using NOD mice model.

They found that in the beginning, MAdCAM-1 expression was very low and was on endothelial cells of the exocrine pancreas, but not on the endothelia within the islet. But as the insulinitis progressed by the age of 4-5 weeks, MAdCAM-1 was detected on the endothelium, within and adjacent to the islet. By week 8, 40 - 44% of islets expressed MAdCAM-1. By week 12, most of the vessels expressed MAdCAM-1. $\alpha_4\beta_7$ -integrins were strongly expressed on the lymphocytes infiltrating the islets at all stages of the insulinitis. It was noted that TK1 cells were more significantly bound to MAdCAM-1 of HEV by $\alpha_4\beta_7$ -integrin as observed in an *in vitro* assay, preincubation of the pancreatic section with anti-MAdCAM-1 and preincubation of cells with anti α_4 and anti- β_7 antibody inhibited the binding (Hanninen et al., 1993).

Other experiments have been performed to test the effect of antibodies against the individual integrins and addressin molecules. Anti-integrin α_4 treatment in the neonatal NOD mice for the first 4 weeks of age exerts significant protection against insulinitis and IDDM (Yang et al., 1994) and treatment with monoclonal antibodies (mAb) for β_7 resulted in prevention of type 1 diabetes (Yang et al., 1997). In addition, treatment of mAb against MAdCAM-1 provided long lasting protection against IDDM in mice (Yang et al., 1997). However, at the same time, these treatments failed to prevent lymphocyte migration to salivary glands (Yang et al., 1997). Authors of both the studies concluded that $\alpha_4\beta_7$ and MAdCAM-1 are involved in tissue selective migration of lymphocytes in type 1 diabetes pathogenesis (Yang et al., 1994 and Yang et al., 1997). In their gut (jejunum) immunohistochemical study, Savilahti et al. (1999) found a significant

increase in $\alpha_4\beta_7$ integrin + mononuclear cells in the LP ($P=0.006$) of type 1 diabetes patients as compared to the control subjects (Savilahti et al., 1999). The observation that higher expression of $\alpha_4\beta_7$ -integrin is induced on the activated T cells in the presence of Th_1 cytokine environment (Abramson et al., 2001) leads to the possibility that gliadin from spingolipid enriched extract might have enhanced expression of $\alpha_4\beta_7$ -integrin on iIELs while inducing autoimmune diabetes.

Chemokine-chemokine receptor pathway

Chemokines are chemoattractant peptides that possess regulatory functions in inflammation, immune cell differentiation and leukocyte trafficking (reviewed by Olson and Ley, 2002). Specific leukocytes (neutrophils, eosinophils, lymphocytes) respond to a particular chemokine depending upon the expression of the appropriate chemokine receptor on its surface (reviewed by Rottman, 1999). Chemokine receptor expression is largely dependent on the cytokine environment. IL-2 induces CCR1, CCR2 and CCR3 expression on T lymphocytes and inhibits via activation through CD3 complex. In addition, IFN- γ induces CCR1 expression and it is also capable of enhancing and suppressing CCR3 and CXCR3 expression depending on T cell polarization (reviewed by Olson and Ley, 2002). On the other hand, IL-4 and IFN- γ respectively up regulate and down regulate CXCR4 expression (reviewed by Olson and Ley, 2002). Moreover Th_1 cells express the chemokine receptors CCR5 and CXCR3. In contrast, Th_2 cells express CCR3, CCR4 and CCR8 (reviewed by Rottman, 1999). In a similar way,

expression of chemokines by IFN- γ is critical for Th₁ lymphocyte, monocyte and neutrophil recruitment. On the other hand, IL-4 and IL-13 induces expression of MCP-1 (Monocyte chemoattractant protein-1), TCA-3 (T cell-activation protein-3) and TARC (Thymus-and activation related chemokine) responsible for Th₂ lymphocyte recruitment (reviewed by Olson and Ley, 2002).

Expressions of multiple chemokines / chemokine receptors have been found in chronic inflammatory diseases such as multiple sclerosis and psoriasis (reviewed by Rottman, 1999). Chemokine and chemokine receptor expression is found to be enhanced in certain autoimmune diseases like multiple sclerosis, an autoimmune disease where the number of CCR5⁺ and CXCR3⁺ T cells is increased and the corresponding ligand (MIP-1 α and IP-1/Mig respectively) expression is also enhanced in brain lesions (Balashov et al., 1999).

Recently, it has become clear that pancreatic chemokines decide the fate of the type 1 diabetes pathogenesis. High relative expression of chemokine macrophage inflammatory protein-1 α (MIP-1 α) and the CCR5 chemokine receptor are found to be associated with the development of insulinitis and autoimmune diabetes whereas high relative expression of MIP-1 β and reduced CCR5 expression in islets resulted in diabetes resistance in NOD mice, thus intrapancreatic MIP-1 α : MIP-1 β ratio is critical deciding factor in either progression or prevention of IDDM (Cameron et al., 2000). Studies have been conducted to investigate the expression of chemokine receptors CXCR4 (naive T associated cells), CCR5 and CXCR3 (Th₁ associated), and CCR3 and CCR4 (Th₂ associated) on peripheral lymphocytes in association of type 1 diabetes.

Th₁-associated chemokine receptors CCR5 and CXCR3 (but not Th₂ or naive T cell associated chemokine receptors) showed a marked decrease in newly diagnosed autoimmune diabetes patients with reduced IFN- γ and TNF- α levels when compared with long standing IDDM patients. Opposite to the chemokine receptors, chemokines MIP-1 α and MIP-1 β levels were found to be elevated in excessiveness in some of the newly diagnosed IDDM patients (Lohmann et al., 2002).

When CCR4 and CCR5 mRNA analysis of the intestinal mucosal samples of pediatric patients with type 1 diabetes and healthy controls were conducted by a reverse transcriptase polymerase chain reaction (RT-PCR) , the patients showed higher intestinal CCR4 expression as compared to healthy controls (Westerholm-Ormio et al., 2003). Thus this study demonstrated the association of type 1 diabetes with a specific small intestinal chemokine pattern (Westerholm-Ormio et al., 2003).

In addition, secondary lymphoid tissue chemokine (SLC) is one of the chemokines crucial to the homing of lymphocytes in the PPs by triggering $\alpha_4\beta_7$ -MAdCAM-1 firm adhesion as was observed in the flow chamber adhesion assay (Pachynski et al., 1998), thus chemokines help in the homing of lymphocytes.

In summary, all these studies indicate a major role of these adhesion molecules and chemokines in lymphocyte influx into the extra-lymphocyte pancreatic islets in type 1 diabetes and $\alpha_4\beta_7$ -MAdCAM-1 as the major path for the extravasations of the diabetogenic lymphocytes from gut to the pancreatic islet.

IX. Th₁-Th₂; Tc₁ -Tc₂ dichotomy and cytokine patterns

Th₁ and Th₂ cytokine production by CD4+T helper cell subsets were first described in a mouse model. Interleukin-2 (IL-2), IFN- γ and lymphotoxin (LT) are produced by murine Th₁ cells, whereas IL-4, IL-5, IL-6, IL-9, IL-10 and IL-13 cytokines are produced by murine Th₂ cells. Although other cells of the immune system generate these cytokines, Th₁ and Th₂ cells are the major sources of their respective cytokines. However, IL-3, TNF- α , GM-CSF (granulocyte-monocyte-colony stimulating factor), and some chemokines are produced by both Th₁ and Th₂ cells types. There are also other phenotypes viz. type T₀ that synthesizes cytokines of both types and a fourth phenotype called Th₃ that produces a high amount of TGF- β (reviewed by Mosmann and Sad, 1996).

These Th₁ and Th₂ subsets are derived from the same precursor naive CD4+ T cell (phenotype-CD44^{low}, CD45RB^{high}, L-selectin) and then expressing memory cell phenotype (phenotype-CD44^{high}, CD45RB^{low}, L-selectin) depending on the nature of the antigen, dose of antigen, co-stimulatory molecules of the APCs and presence of the cytokines (reviewed by Mosmann and Sad, 1996). Initially CD4+ T cells are the naive cells producing IL-2 cytokine alone. When these cells encounter antigens, they get stimulated and acquire memory phenotype and finally, depending on the presence of IL-4 or TGF- β at that time, they then differentiate into respective Th₁ and Th₂ subtypes (reviewed by Mosmann and Sad, 1996). In Th₁ cell type development and differentiation, IL-12 produced by APCs, signal transducer and transcriptional factors- signal

transducer and activator of transcription 4 (STAT4) and T-bet play an essential role (reviewed by Seder and Ahmed, 2003). Importantly, IL-12 produced by dendritic cell after antigen-specific T cell interaction was shown to be important for the optimal proliferation of Th₁ cells and subsequent IFN- γ production by these activated Th₁ cells (Heufler et al., 1996). On the other hand, IL-4, in conjunction with STAT6 and the transcription factor GATA-3 (trans-acting T-cell specific transcription factor), are important for Th₂ type development (reviewed by Seder and Ahmed, 2003).

Also the cytokine synthesized by these subsets further influences Th₁-Th₂ differentiation. IL-4 enhances the differentiation of naive CD4⁺ T cells into Th₂ type whereas IFN- γ , IL-12 and TGF- β encourage the Th₁ type differentiation (reviewed by Mosmann and Sad, 1996). Another important characteristic of these Th₁ and Th₂ patterns is that their cytokine uptake has the potential to switch or inhibit cells into a different pattern of secretion (reviewed by Mosmann and Sad, 1996).

Considering their role, Th₁ cells with their distinctive cytokine profile, are involved in cell mediated inflammatory responses and cytotoxic activation whereas Th₂ cells, with their cytokines, are responsible for the antibody production and its responses (especially IgE) and thus Th₂ cells contribute to allergic reactions. Th₁ and Th₂ cytokine biases are found in number of autoimmune diseases (reviewed by Mosmann and Sad, 1996).

Similar to CD4⁺ helper cell subsets, subsets of CD8⁺ T cells, cytotoxic cells (Tc), Tc₁ and Tc₂ are identified in murine model and in humans as well.

Although it was initially thought that CD8+ T cells often secrete a Th₁ like cytokine pattern, it is now clear that CD8+ T cells also possess another type called Tc₂. Analogues to the Th subset, IL-12 and IFN-γ, encourage the differentiation of precursors into Tc₁ cells, whereas IL-4 induces the generation of Tc₂ cells (reviewed by Mosmann and Sad, 1996). Another characteristic of distinct CD8+ T cell subsets is the irreversible differentiation into either of the two types, once they are differentiated into Tc₁-Tc₂, they do not convert into other cytokine secretion patterns. Tc₁ subsets strictly possess the cytotoxic function, whereas Tc₂ subsets exhibit a cytotoxic function only in some systems (reviewed by Mosmann and Sad, 1996).

CD4+T cells with regulatory phenotype and their role in autoimmune disease

CD4+T cells could be also divided in two subsets depending on the expression of CD45RB expression i.e- CD45RB^{high} and CD45RB^{low}. These cells subset decides the fate of a number of inflammatory diseases (reviewed by Shevach, 2000). Injecting CD4+/CD45^{high} to congenic immunodeficient SCID mice resulted into development of wasting syndrome, increase splenic and lymph node cellularity. It also induced drastic hyperplasia and enhances lymphocytic infiltration in the intestine when compared to the SCID mice injected with CD4+/CD45^{low} cell type. In addition, a five fold increase in CD45^{high} cell type was observed indicating proliferation of this cell type in the recipient. The author further attributed their respective pathological and immunological roles to the

different cytokines produced by these cells after stimulation (Morrissey et al., 1993). For example, enhanced mRNA expressions of IFN- γ without affecting the mRNA expression of IL-4 and IL-10 in the disease specific tissues were observed in colitis in SCID recipient of CD45RB^{high} cells. On the other hand, transfer of CD4⁺ CD45RB^{low} cells completely inhibited diabetes and insulinitis (reviewed by Shevach, 2000). Thus CD4⁺T cells with regulatory or CD45RB phenotype decides the autoimmune manifestations in several diseases (reviewed by Shevach, 2000).

In another study, colitis and wasting disease induced by CD4⁺/CD45^{high} cell in SCID mice were found to be ameliorated by CD4⁺CD25⁺ regulatory cell treatment. These regulatory T cells were found to be located at the interface of the APC and effector cells which suggests that these cell types interact with APCs and interfere with their ability to activate the effector cells (Mottet et al., 2003). Recently, Sarween et al.(2004) have demonstrated that CD4⁺CD25⁺ T cells successfully prevented type 1 diabetes development in an adoptive transfer based model of type 1 diabetes. They found that these regulatory T cells significantly inhibited islet infiltration by pathogenic CD4⁺ T cells. These regulatory T cells have found to exert this protective effect by reducing IFN- γ secreting cells and thus interfering IFN- γ dependent chemokine receptor pattern (Sarween et al., 2004).

Th₁-Th₂ cytokine biases in type 1 diabetes

Th₁-Th₂ biases have been found in autoimmune diseases including IDDM (reviewed by Mosmann and Sad, 1996). Adoptive transfer of Th₁ cells and clone lines in neonatal NOD mice have shown to home to the pancreas, and are capable of inducing IDDM, whereas Th₂ cells induce nondestructive peri-islet insulinitis, which suggests that type 1 diabetes is a Th₁ mediated disease (Healey et al., 1995). Importantly, these two subsets render their function via their cytokine profiles (Healey et al., 1995). Ng et al (1999), observed most of the IDDM patients (63.4%) had at least one of the Th₁ cytokines i.e- IFN- γ , TNF- α , IL-12 detected as compared to a few of them (9.75%) those had detectable Th₂ cytokine profile i.e.-IL-4 when the investigators conducted sensitive ELISA assay on the sera procured from IDDM patients (Ng et al., 1999).

On the other hand, there is a large body of evidence concerning the protective role of Th₂ cytokines in IDDM. IL-4 administration to prediabetic mice has been shown to prevent the onset of the disease in NOD mice (Rapoport et al., 1993). This could be because IL-4 has effect on Th₂ cells establishment. Pancreatic expression of IL-4 has been observed to enhance the Th₂ environment, which inhibited the action of diabetogenic lymphocytes and successfully maintained the nonpathogenic stage in IDDM (Gallichan et al., 1999).

Most importantly the Th₁/Th₂ ratio is critical to disease fate. Berman et al. (1996) have shown that type 1 diabetes is characterized by an aberrant Th₁/Th₂ ratio with impaired production of IL-4, which is not due to impaired production of

IFN- γ , leading to an imbalance of inflammatory vs anti-inflammatory cytokine production resulting in disease pathogenesis of the disease (Berman et al., 1996).

Interestingly, a study conducted with IFN- γ NOD SCID recipient and IL-4 NOD SCID recipient demonstrated that the pancreatic Th₁ cytokine environment induces type 1 diabetes by accelerating the recruitment of adoptively transferred islet specific CD4⁺ cells to the pancreas. Pancreatic Th₂ environment, however, suppresses the disease because of a decreased rate of recruitment of islet specific T cells resulting in a smaller number of diabetogenic T cells in the pancreas. Thus Th₁ cytokine production by initially infiltrated islet cells determines the further recruitment of the activated T cells in the pancreas and the acceleration of onset of the disease. This is possibly due to the ability of these cytokines to alter the local expression of chemoattractant and adhesion molecules (Hill et al., 2003). In various gut associated diseases like in celiac disease and even in infection, LPLs' responses are mostly skewed to a Th₁ phenotype (reviewed by MacDonald et al., 1999).

X. Cytokines IFN- γ and TNF- α concentration change in dietary diabetogen induced type 1 diabetes.

In BBdp rats, IFN- γ and TNF- α are Th₁ cytokine and proinflammatory cytokines respectively (reviewed by Rabinovitch, 1998). IFN- γ is primarily synthesized by Th₁ (CD4⁺) cells, whereas TNF- α is primarily synthesized by

macrophages (reviewed by Rabinovitch, 1998). Both of these cytokines participate in and help regulate cell mediated immunity i.e. cytotoxicity and inflammatory responses by T cells in autoimmune diseases. IFN- γ , synthesized by infiltrating lymphocytes and macrophage derived TNF- α , are directly cytotoxic to pancreatic β cells in IDDM (reviewed by Rabinovitch, 1998). Both of these cytokines are found to activate JNK/SAPK (*c-Jun* NH₂ terminal kinase) synergistically and thus induce Beta cell apoptosis through a p53 pathway with ROS activation and induce apoptosis of pancreatic β cells (Kim et al., 2005). These cytokines also induce expression of MHC class I molecules and Fas receptor molecules on the pancreatic β cells which makes them susceptible to the lymphocytic attack (reviewed by Rabinovitch, 1998). When gut immune system is considered, LPLs notably secrete IFN- γ (reviewed by MacDonald et al., 1999).

Cytokine IFN- γ and type 1 diabetes

The positive correlation between the pancreatic expression of Th₁ cytokines and destruction of islet β cells in BBdp rats and NOD mice has been well documented (reviewed by Rabinovitch, 1998). Mononuclear cell infiltration in the pancreas and upregulation of MHC class I or class II molecules do not occur in transgenic mice those are genetically deficient in IFN- γ , thus protecting the mice from IDDM (von Herrath and Oldstone, 1997). Additionally, enhanced IFN- γ mRNA expression in islet infiltrating lymphocytes (Rabinovitch et al., 1996) and in

the pancreas (Schloot et al., 2002) is essential for the β cell destructive insulinitis and IDDM.

In addition IDDM severity is dependent on the relative frequency of T lymphocytes capable of secreting IFN- γ upon activation as observed in NOD mice model (Ablamunits et al., 1999). In humans, ~ 40% of the lymphocytes were found IFN- γ positive when Foulis et al. (1991) studied 87 islets of the autopsied pancreata of 12 type 1 diabetes patients (Foulis et al., 1991). Nicoletti and colleagues (1997) reported that anti-IFN- γ mAb treatment at the doses of 100 and 200 μg /week from the 30/33th to 105th days of age of the BB rats reduced diabetes incidence and delayed the onset age of the disease (Nicoletti et al., 1997).

In addition to IFN- γ producing CD4+, IFN- γ producing CD8+ cells have been found to be responsible for the β cell destruction and recurrence of the disease in syngeneic pancreatic islet grafts in diabetic NOD mice, which revealed the importance of the IFN- γ produced from CD8+ cells in the pathogenesis of the disease (Suarez-Pinzon et al., 1996).

The primary mechanism by which IFN- γ mediates type 1 diabetes is through IFN- γ induction of hyper expression of the class I MHC molecule on the pancreatic β cells making pancreatic β cells prone to destruction (reviewed by Rabinovitch, 1998). Secondly, IFN- γ plays an important role in autoimmune diabetes because pleiotropic function has been exhibited by this cytokine that influences the homing of lymphocytes to the pancreas. Although adhesion of insulin-specific CD8+ cells to microvasculature was found normal in IFN- γ -KO

(Interferon gamma knock out) or in NOD mice lacking beta-chain of IFN- γ receptor, significant impairment in the diapedesis was observed affecting the homing of these lymphocytes to the pancreatic islet, thus resulting in inefficient adoptive transfer of diabetes (Savinov et al., 2001). Thus the results of this study prove that IFN- γ is critical for IDDM pathogenesis.

Cytokine TNF- α and type 1 diabetes

Yang et al. (1994) have demonstrated a crucial role for TNF- α in IDDM development, especially in early development of autoimmunity towards β cells (Yang et al., 1994). Overt diabetes incidence was observed 4 weeks earlier in TNF- α treated neonatal NOD mice (3 week treatment) as compared to control mice, whereas by 20 weeks all of the TNF- α administered mice developed IDDM. On the other hand, treatment with anti-TNF- α mAb was found to exert a protective effect on the onset of IDDM development (Yang et al., 1994).

The important role of TNF- α in the early stage of the disease development has been revealed from the results of a study by Christen et al. (2001). They observed enhancement of β cell destruction and IDDM by islet-specific expression of TNF- α and complete prevention of the disease by blocking of TNF- α in a transgenic model of type 1 diabetes at an early stage of disease development (Christen et al., 2001). TNF- α genes are expressed during the disease progression with TNF- α mRNA-positive cells have been found exclusively present in the intra-islet infiltrate. Importantly TNF- α mRNA positive CD4⁺ T cells were found in close vicinity to pancreatic β cells which suggests

TNF- α specific lymphocytes may destroy the β cells in type 1 diabetes (Held et al., 1990).

Cytokine TNF- α takes part in the pathogenesis of IDDM by various mechanisms. TNF- α enhances islet infiltrating APCs to cross present exogenous antigens to CD8+T cells by a unique path (Green et al., 2000). TNF- α may also increase the adhesion molecule expression on endothelial cells of the venules found on islets, thus directly helping in the process of homing of T cells to the islets as observed in a experiment conducted with cultured endothelial cells (Doukas and Pober, 1990).

Collectively, IFN- γ and TNF- α cytokines possess potential to destroy pancreatic β cells possibly through free radical formation. Indeed an *in vivo* study showed that IFN- γ , TNF- α and IL-1 β together lead to free radical formation when they administered IFN- γ , TNF- α and IL-1 β to rat pancreata directly (Tabatabaie et al., 2003). Also these cytokines synergistically influence the expression of adhesion molecules on the endothelial cells (Doukas and Pober, 1990) affecting lymphocyte trafficking in the pathogenesis of type 1 diabetes.

Diet modifies cytokine IFN- γ and TNF- α in type 1 diabetes

Cytokine expression and synthesis pattern has found to be influenced by diet. In BB rats, the NIH diet was found to be associated with an increased expression of IFN- γ and low expression of IL-10, TGF- β in the pancreas, deviation to the Th₁ cytokine pattern (Scott et al., 1997). Whereas pancreata of hydrolyzed casein diet fed rats were found to be associated with the low IFN- γ

expression and high expression of TGF- β , the deviation to the Th₂ cytokine pattern (Scott et al., 1997). Recently a wheat based NTP 2000 diet was reported to induce significantly greater expression of Th₁ cytokine and pro inflammatory markers, IFN- γ , TNF- α , and inducible NO synthase mRNA in the small intestine of NOD mice as compared to Prosobee and Prosobee + casein diet, whereas Th₂ cytokine IL-10 and TGF- β expression was found to be unaffected (Flohe et al., 2003). Cytokine modulating properties of the CM soluble gliadins from sphingolipid enriched extract might have played a role in disease pathogenesis in our study. Gliadin's proteolytic fragment is able to elicit significant production of TNF- α in human monocytic line THP-1 cells (stages of monocyte/macrophage maturation) (Jelinkova et al., 2004). In addition IFN- γ was found to act synergistically with gliadins while inducing gliadin induced TNF- α secretion (Jelinkova et al., 2004).

Serum IFN- γ and TNF- α levels in type 1 diabetes

The serum IFN- γ level has been found to be correlated with the IDDM pathogenesis with highest serum IFN- γ levels at the time of disease onset in NOD mice (Schloot et al., 2002). Similarly serum TNF- α levels (> 1U/ml) were detected in ~24% of IDDM patients at the time of diagnosis but TNF- α levels were not detected in long standing diabetes patients, suggesting the role of elevated TNF- α level in early IDDM pathogenesis as well (Cavallo et al., 1991). In addition, it has been found that 63.4% of type 1 diabetes patients have at least one of the proinflammatory Th₁ systemic (serum) cytokine TNF- α , IFN- γ , IL-12 level detectable. In contrast only 9.75% of patients have systemic (serum) Th₂

type cytokine- IL-4 detectable, which suggests a dominant Th₁ cytokine pattern in IDDM (Ng et al., 1999). When serum cytokines IFN- γ , TNF- α levels between recently diagnosed IDDM patients and long standing IDDM patients were investigated, serum cytokine IFN- γ , TNF- α levels were found to be significantly elevated in recently diagnosed type 1 diabetes patients as compared to long standing IDDM, suggesting the importance of serum cytokine levels in the early stages of the disease development (Hussain et al., 1996). Thus elevated serum IFN- γ and TNF- α may be responsible for the IDDM pathogenesis and development.

In summary, the CM soluble gliadins specific sphingolipid enriched extract may have induced type 1 diabetes by activating the gut immune system and changing cytokine levels in a series of events with a wide array of interactions between pancreatic β cells, lymphocytes, MHC class molecules, adhesion molecules, chemokines and cytokines etc.

Chapter 3. Detection of Gliadin Proteins in Chloroform Methanol Soluble Extract of Wheat Gluten and Sphingolipid Enriched Extract by SDS-PAGE.

Abstract

Earlier wheat α and γ gliadins were shown to be CM soluble. Therefore to assess the presence of wheat α and γ gliadins in CM soluble sphingolipid enriched extract, a protein assay and SDS-PAGE analysis of CM soluble extract of wheat gluten and CM soluble sphingolipid enriched extract was conducted. For CM soluble wheat gluten extract, a electrophoretic bands approximately at 41 kD and 12 kD were obtained whereas the CM soluble sphingolipid enriched extract exhibited diffused band patterns between \sim 15 kD-120 kD. The SDS-PAGE electrophoretic band patterns of CM soluble extract of wheat gluten and CM soluble sphingolipid enriched extract correspond to the molecular weights of wheat α and γ gliadins. In addition, the diffused band pattern observed for CM soluble sphingolipid enriched extract could be attributed to the presence of lipids in the CM soluble sphingolipid enriched extract. Thus, the results of SDS-PAGE analysis suggest the presence of wheat α and γ gliadins in the CM soluble sphingolipid enriched extract.

Introduction

The results of the Shi, 2003 study (M.S. Thesis) regarding the diabetogenic potential of sphingolipid were ambiguous since the ultimate diabetes incidence was not influenced by either the sphingolipid enriched extract or the residue remaining after CM extraction.

Recent literature suggests that α -gliadin and γ -gliadins are CM soluble proteins (Rocher et al., 1995). Moreover, gliadins have proven to increase type 1 diabetes incidence (Elliott and Martin, 1984). Increase in antigliadin antibodies have been reported in type 1 diabetes patients with normal jejunal biopsy (absent of celiac disease patients), especially at the onset of IDDM (Carlsson et al., 1999). Auricchio et al., (2004) also reported of gliadin specific mucosal immune responses in type 1 diabetes (Auricchio et al., 2004). Thus, all these studies lead to the speculation that the CM soluble sphingolipid enriched extract contains gliadin proteins that possess diabetogenic potential. To test the presence of gliadin proteins in the sphingolipid enriched extract, which was used in the feeding study (Shi, 2003-M.S. Thesis, Virginia Tech), we conducted a retrospective sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the CM soluble extract of wheat gluten and the sphingolipid enriched extract stored from the Shi, 2003 study (M.S. thesis).

Materials and Methods

Materials

The CM soluble wheat gluten extract and sphingolipid enriched extract for the protein assay and SDS-PAGE were obtained from Shi, 2003 study (M.S. Thesis).

Chemicals and Reagents

BSA protein assay reagent kit and GelCode Blue Stain reagent solution (Prod # 24590) were purchased from PIERCE. Ready gels, 10X Tris-Glycine buffer, Laemmli sample buffer and precision plus proteinTM standard with known molecular weights were bought from BIO-RAD.

SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis)

First a protein assay to quantify the proteins in the CM soluble extract and the sphingolipid enriched extract were conducted as per manufacturer's recommended protocol with few modifications (Pierce, Rockford, IL, USA). To prepare the sample, the CM soluble extract of wheat gluten / sphingolipid enriched extract was mixed with extraction buffer and homogenized with the homogenizer (VIRTIS-VIRTISHEAR, The Virtis Company Inc. NY). The homogenized mixture was then incubated for 2 hour at 65 °C in water bath. After incubation, the mixture was centrifuged, and the supernatant was used for the protein assay using the test tube procedure of BCATM protein assay. BSA was used as a standard. BSA working reagent was added to the samples and the

absorbance of the resulting mixture was read by using a spectrophotometer at 562 nm. The protein concentrations were determined based on a standard curve.

After estimating the amount of protein in the CM soluble extract of wheat gluten and the sphingolipid enriched extract by protein assay, proteins were separated by using SDS-PAGE. The SDS-PAGE was performed as described by Laemmli's (1970) with minor modifications (Laemmli, 1970). First, samples of the CM soluble extract of wheat gluten and the sphingolipid enriched extract were prepared in the same way as for the protein assay. Finally samples for SDS-PAGE analysis were prepared by adding 50 μ l of the non-reducing sample buffer to 50 μ l of the CM soluble wheat gluten and the sphingolipid enriched extract samples. The resulting mixtures were loaded onto the ready gel (18% Tris-HCl, 10 well and 30 μ l comb) in separate wells and subjected to electrophoresis on mini-acrylamide gels using Mini-Protein III (BIO-RAD) apparatus. Electrophoresis was performed along with protein markers at 180V, 100 mA for 50 minutes at room temperature.

Then the gel was washed with ultrapure water and immediately stained with GelCode Blue Stain Reagent solution for 1 hour. Then ultra pure water was used for destaining. Finally the gel was scanned by using a scanner Alpha Image 2000 connected to a computer, which was equipped with Alpha Imager 2000 3.3 b software (Alpha Innotech Corporations). The molecular weights of the resulting protein bands were determined in kD by comparing with the molecular weight of marker proteins. The resulting band intensities were also compared to assess the protein content in the respective extract samples.

Results

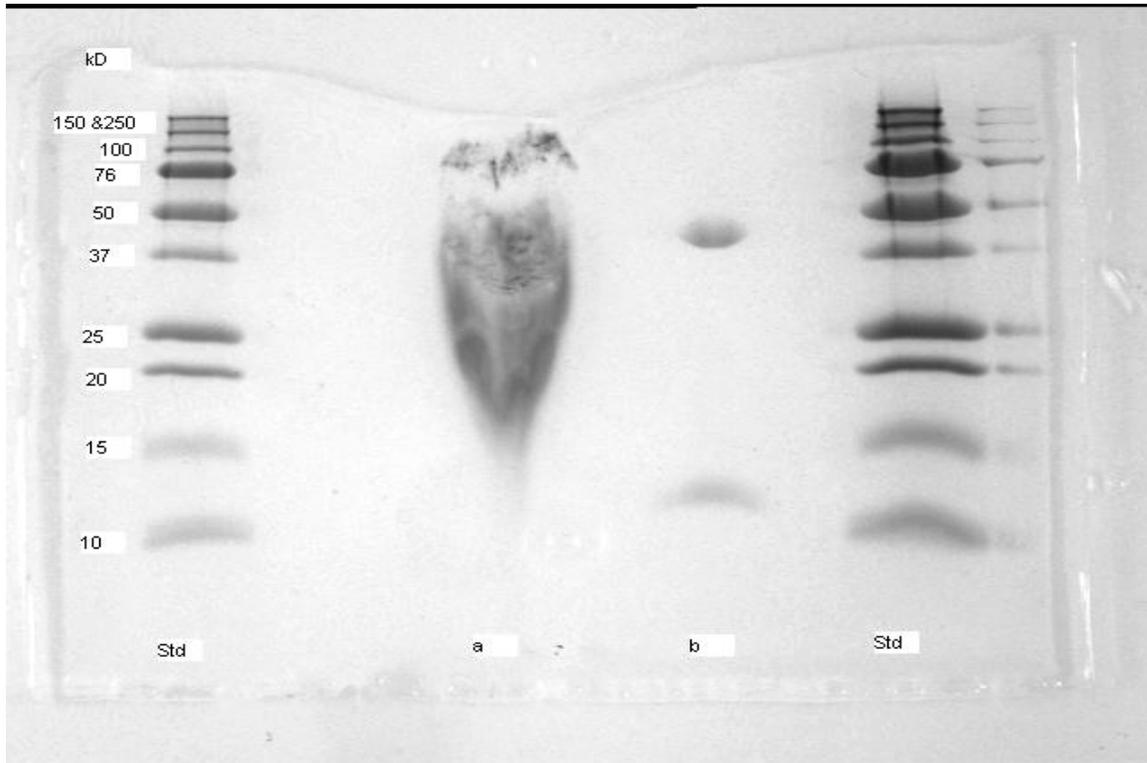


Figure 1. SDS-PAGE analysis of non-reduced chloroform-methanol (CM) soluble extract of wheat gluten and sphingolipid enriched extract. Lane a represents the sphingolipid enriched extract; Lane b represents the CM soluble extract; Lane Std represents the protein reference with known molecular weight; Molecular weights are in kD as shown in the figure.

The results of the SDS-PAGE analysis of the CM soluble extract of wheat gluten and the sphingolipid enriched extract are shown in the Figure 1. For the CM soluble extract of wheat gluten, two bands with approximate molecular weights of ~41 kD and ~12 kD were obtained (Figure 1 Lane b.). The sphingolipid enriched extract did not show a distinct electrophoresis pattern, however diffused bands were observed with molecular weights ranging between 15 kD-120 kD (Figure 1 Lane a.).

Discussion

The electrophoretic pattern consisting of two distinct bands of molecular weight of ~41 kD and ~12 kD were obtained when the CM soluble extract of wheat gluten was analyzed by SDS-PAGE. Whereas bands with molecular weights corresponding to a range between 15 kD - 120 kD were observed when the sphingolipid enriched extract was analyzed by SDS-PAGE. Electrophoretic bands of sphingolipid enriched extract seem to be diffused, almost merged giving the appearance of a smear of proteins. The diffused bands showed high intensity corresponding to 20 kD-50 kD molecular weights.

Earlier findings that the mass ranged of CM soluble gliadin proteins from 15 kD to 50 kD (Rocher et al., 1995) strengthens our hypothesis about the presence of α and γ gliadins in the CM soluble extract of wheat gluten and in the sphingolipid enriched extract. SDS-PAGE analysis of the chromatographic fraction obtained after RP-HPLC of the CM soluble extract of wheat endosperm,

followed by immunoblotting with coelic serum and finally microsequencing resulted into distinct pattern with two groups as observed by Rocher et al., (1995). One group consisted of components with a mass of 15 kD-20 kD and the second group had components with a mass range of 30 kD-50 kD (Rocher et al., 1995). The results of their study showed that most of the CM soluble α and γ gliadins possess masses between 31 kD-45 kD (Rocher et al., 1995).

Recently, Mamone et al. (2005), found α -gliadin peptides (chymotryptic peptides) with masses between 15 kD-35 kD. Whereas γ -gliadin peptides have masses ranging from ~5 kD and ~29 kD (Mamone et al., 2005). In addition, Prabhasankar (2002) found a common band of 40 kD for the gliadin when 10 wheat varieties were analyzed by SDS-PAGE. Also distinct electrophoretic pattern with bands ranging from ~5 kD to 94 kDa were observed for gliadins (Prabhasankar, 2002). Importantly, Friis et al., (1994) showed that crude gliadin containing α and γ gliadins which has dominant bands between 30 kDa and 48 kDa on SDS-PAGE (Friis et al., 1994). Thus these studies support our findings suggesting the presence of gliadins in the sphingolipid enriched extract.

The electrophoretic pattern of the sphingolipid enriched extract showed an upper mass limit of ~120 kD. In the presence of lipids, HMW and LMW polypeptides in gliadin have a tendency to form aggregates with high molecular weights (Lasztity, 1995).

Diffusion of the band could be attributed to the physically complexed lipid-protein (Huang et al., 2006) in the sphingolipid enriched extract. Additionally, Barbeau and Kinsella. (1987) reported diffused band patterns of proteins on

SDS-PAGE, and they attributed this altered electrophoretic pattern to the presence of a large amount of fat in the protein sample (Barbeau and Kinsella, 1987). Thus the electrophoretic pattern of wheat gliadin proteins from the sphingolipid enriched extract could be attributed to the presence of a large amount of lipids in the sample as compared to proteins. Treating the CM soluble sphingolipid enriched extract sample with chloroform methanol mixture to remove lipid fraction from the samples prior to SDS-PAGE analysis was not employed in our experiments as described by Huang et al. (2006) (Huang et al., 2006).

From the intensity of the electrophoretic bands, it is very clear that both extracts contained a low amount of gliadin proteins. The faint black colored band may be due to the presence of lipid portion accompanying the proteins in both the samples since completely defatted sample exclusively containing proteins usually gives dark colored bands on SDS-PAGE (Sringam et al., 1997). Furthermore, the resolution of the electrophoresis pattern might have improved with silver staining as used by Prabhasankar, (2002) (Prabhasankar, 2002), instead we used GelCode Blue Stain reagent for staining.

Collectively, our findings suggest the presence of gliadin proteins in the sphingolipid enriched extract.

Chapter 4. Assessment of Insulinitis and Histopathology of Pancreata of BBdp Rats Fed 5 Different Diets : Role of Chloroform Methanol Soluble Gliadin Specific Sphingolipid Enriched Extract.

Abstract

Diet influences the type 1 diabetes incidence and insulinitis score. To assess the effect of five different dietary treatments on the insulinitis score especially lymphocytic infiltration in the islet of Langerhans and pancreatic lymphoid aggregate content, a microscopic evaluation of H & E stained tissue slides of pancreata of BBdp rats fed five different diets were conducted. Significantly higher insulinitis scores were observed in BBdp rats from NTP 2000 dietary treatment when compared to BBdp rats from HC and HCGSL dietary treatment group. There were no significant differences observed in insulinitis score when BBdp rats from HC and HCGSL dietary treatment groups were compared. Also there were no significant differences observed in insulinitis score in BBdp rats fed NTP 2000 diet vs WG; WGGSLG diet. However WG dietary treatment was associated with increase in insulinitis score when compared to WGGSLF, although the increase was not significant. For pancreatic lymphoid aggregate content, we did not find significant differences in BBdp rats between any two dietary treatment groups. These findings demonstrate that NTP 2000 and WG diet accelerate the insulinitis in BBdp rats whereas HCGSL diet suppresses it.

Introduction

At the clinical onset of the type 1 diabetes, the islet of Langerhans is usually deficient in pancreatic β cells with 80% of the pancreatic β cells destroyed by that time (reviewed Atkinson and Maclaren, 1994). The resulting islet mostly consists of cells with enlarged nuclei and degranulated β cells with an inflammatory infiltrate (reviewed Atkinson and Maclaren, 1994). This mononuclear cell infiltration in the islet is called insulinitis (reviewed by Bach, 1994). Insulinitis occurs in various stages during disease progression viz. peri-insulinitis (mononuclear cell infiltrate around the islets), peripheral insulinitis (lymphocytes at the islet periphery), destructive and invasive insulinitis (reviewed by Bach, 1994). By the time ~100% β cells are destroyed, the insulinitis disappears giving a small, distorted and atrophied islet, a characteristic histological feature of islets of patients with long standing disease (reviewed Atkinson and Maclaren, 1994). For instance, Foulis et al.(1986) reported presence of insulinitis in 78% of the patients with recent onset of the diabetes vs in only ~8% of long standing diabetes patients. In essence, insulinitis is a characteristic feature of the recent onset of type 1 diabetes (Foulis et al., 1986).

Published literature suggests that diet modifies type 1 diabetes incidence in experimental animal models. Therefore several groups investigated the effect of diet on the insulinitis score and islet morphology in animal model of type 1 diabetes. Hoorfar et al. (1993) studied the effect of diet on the frequency of insulinitis and they observed less frequent insulinitis in NOD mice fed hydrolyzed casein diet as compared to NOD mice on CBD (wheat flour) or hypo-allergenic

diet-soybean protein hydrolysate (Hoorfar et al., 1993). Wang et al. (2000) scored insulinitis with evaluation of islet neogenesis and islet area in BBdp and BB control rats fed hydrolyzed casein and NIH-07 to assess the mechanism of diet induced type 1 diabetes. They found less insulinitis and significantly higher insulin clusters in animals fed HC diet as compared to the animals fed NIH diet (Wang et al., 2000).

Most of the studies in this direction fed the CBD, hydrolyzed casein diet or hypoallergenic diet to the animal model of type 1 diabetes. No studies have addressed the effect of the CM soluble gliadin specific sphingolipid enriched extract (GSLEE) on the pancreatic histopathological parameters, insulinitis scores i.e lymphocytic infiltration in islet of Langerhans and pancreatic lymphoid aggregate contents. The effect of diet on adipose tissue content has not been studied either. Therefore, as a follow up study to Shi, 2003 (M.S. Thesis) study, we determined the effect of 5 different dietary treatments on insulinitis score, lymphoid tissue aggregates and adipose tissue content.

Since SDS-PAGE analysis of the CM soluble extract of wheat gluten and the sphingolipid enriched extract suggested presence of gliadin proteins in the CM soluble extracts. Therefore we named HCSL diet (Hydrolysed casein based diet with the sphingolipid enriched extract added to it) and WGSLF diet (Wheat gluten based with the sphingolipid enriched extract removed from the diet) from the Shi, 2003 study (M.S. Thesis) as HCGSL diet (Hydrolysed casein based diet with the gliadin specific sphingolipid enriched extract added to it) and WGGSLF

diet (Wheat gluten based with the gliadin specific sphingolipid enriched extract removed from the diet) respectively.

Materials and Methods

Material

Table 3. Total number of H&E stained pancreatic tissue slides from 5 different dietary treatment groups.

Abbreviations for the dietary feeding group	Diet	Number of BBdp rats with H&E stained pancreatic tissue specimen slides
A	HC	20
B	NTP 2000	19
C	WG	18
D	WGGSLF	17
E	HCGSL	17
		Grand total = 91

1. HC-Hydrolyzed casein based diet, which is the negative control.
2. NTP 2000-National toxicology program 2000 diet, which is the positive control.
3. WG- Wheat gluten based diet.
4. WGGSLF- Wheat gluten based with the gliadin specific sphingolipid enriched extract removed from the diet.
5. HCGSL- Hydrolyzed casein based diet with the gliadin specific sphingolipid enriched extract added to it.
6. A, B, C, D, E - Abbreviations used for the respective dietary treatments.

Haematoxylin and Eosin (H&E) stained pancreatic tissue slides of the BBdp rats from 5 different dietary treatments from the Shi, 2003 study (M.S.

Thesis) were obtained from Dr. Scott's Laboratory, Ottawa Health Research Institute, Ottawa Hospital, General Campus, Canada as enlisted in Table 3. These slides were stored at 4 °C until used for analysis.

Scoring of insulinitis and tissue histopathology parameters

All the H& E stained pancreatic tissue slides were observed under a microscope (Nikon, ECLIPSE ε400) for insulinitis, lymphocyte/neutrophil prominence, contents of lymphoid aggregates and adipose tissues and histological features like distorted or atrophied islets. Islets were evaluated for insulinitis on the scale of 1 to 5 with 1→ mild mononuclear cell infiltration (granulation 1%-20% of the total islet), 2→ moderate mononuclear cell infiltration (granulation 21%-40% of the total islet), 3→ moderate-severe mononuclear cell granulation (granulation 51%-60% of the total islet), 4 → severe mononuclear cell infiltration (granulation 61%-80% of the total islet) and 5 → severe massive mononuclear cell infiltration (granulation 81%-100% of the total islet) using 400X (10X ocular and 40X objective) magnification. Five microscopic fields of islets were observed randomly and the average was calculated for the insulinitis score for that particular pancreatic tissue section of the BBdp rat. Lymphoid aggregate content in the H&E stained pancreatic tissue slide were scored on a scale of 1 to 3 with, 1→ less content, 2 → medium content and 3→ high content of adipose tissue and lymphoid aggregates, by using 200X magnification. Pancreatic adipose tissue content was also scored on the scal of 1 to 3 at 200X magnifications.

Statistical Analysis

The data from a pancreatic tissue histopathology were analyzed by one way-ANOVA (analysis of variances) using the statistical analysis software (SAS) software version 9.1 (SAS Institute Inc. Cary, NC, USA). General linear model procedures of SAS were used to perform ANOVA. The differences between means were determined by scheffe's multiple comparisons test. Adjusted means were least square means. Differences were considered significant if $p \leq 0.05$.

Results

Table 4. Effect of dietary treatments on the histopathological parameters of pancreas.

Dietary treatments	N	Insulinitis score	Lymphoid aggregate content
HC	20	2.42 ± 0.17 ^c	0.85 ± 0.30 ^a
NTP 2000	19	3.39 ± 0.18 ^a	1.52 ± 0.31 ^a
WG	18	3.26 ± 0.18 ^{ab}	1.22 ± 0.32 ^a
WGGSLF	17	2.64 ± 0.19 ^{ac}	1.17 ± 0.32 ^a
HCGSL	17	2.36 ± 0.19 ^c	1.64 ± 0.32 ^a

1. All values were reported as LSMEAN ± Std Err LSMEAN and were analyzed by ANOVA by using GLM.
2. n represents the number of BBdp rats in respective dietary treatment.
3. HC represents hydrolyzed casein based diet, which is the negative control.
4. NTP 2000 represents national toxicology program 2000 diet, which is the positive control diet.
5. WG represents wheat gluten based diet.
6. WGGSLF represents wheat gluten based with the gliadin specific sphingolipid enriched extract removed from the diet.
7. HCGSL represents hydrolyzed casein based diet with the gliadin specific sphingolipid enriched extract added to it.
8. There were significant differences ($P \leq 0.05$) when groups were marked by different letters.

The insulinitis score and lymphocyte aggregate were two parameters studied while performing pancreatic histopathological analysis. The results of the histopathological analysis of pancreatic tissue specimens are listed in Table 4. The HC and HCGSL diet group's BBdp rats have significantly lower insulinitis scores than the NTP 2000 and WG diet group's rats. No significant differences in insulinitis scores of BBdp rats of NTP 2000, WG, WGGSLF dietary treatment

groups were obtained. Similarly, there were no significant differences in insulinitis score in BBdp rat from the HC and HCSL dietary treatments.

Islet infiltration was found around the periphery as well as in the interior of the islet as well as shown in Figure 2. Lymphocyte prominancy was observed in infiltrated islets and none of the islets were distorted or atrophied.

There were no significant differences between any two groups among all the five diets, considering pancreatic lymphocyte aggregates content.

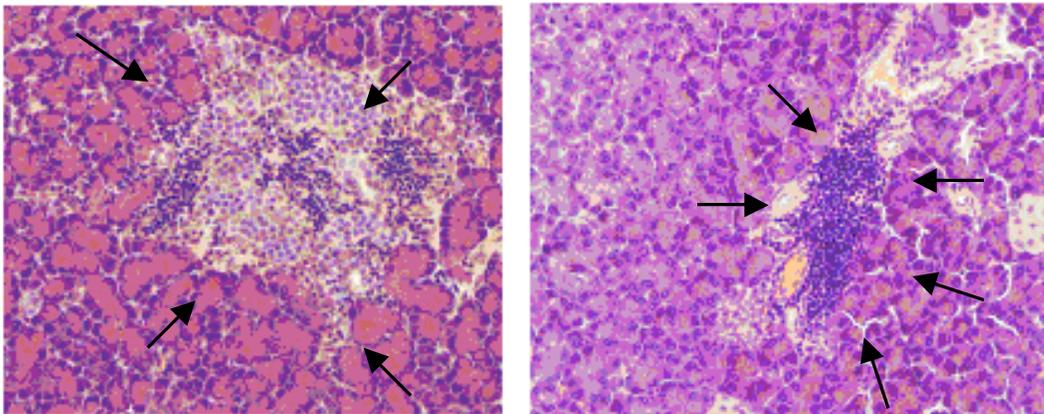


Figure 2. H&E stained pancreatic tissue sections from BBdp rat showing insulitis. Islet infiltration is around the periphery and in interior as well. Original magnifications 200X.

Table 5. Effect of dietary treatments on the pancreatic adipose tissue content.

Dietary treatments	n	Adipose tissue content
HC	20	1.90 ± 0.20 ^a
NTP 2000	19	1.47 ± 0.20 ^a
WG	18	1.50 ± 0.21 ^a
WGGSLF	17	2.11 ± 0.22 ^a
HCGSL	17	2.05 ± 0.22 ^a

1. All values were reported as LSMEAN ± Std Err LSMEAN and were analyzed by ANOVA by using GLM.
2. n represents the number of BBdp rats in respective dietary treatment.
3. HC represents hydrolyzed casein based diet, which is the negative control.
4. NTP 2000 represents national toxicology program 2000 diet, which is the positive control diet.
5. WG represents wheat gluten based diet.
6. WGGSLF represents wheat gluten based with the gliadin specific sphingolipid enriched extract removed from the diet.
7. HCGSL represents hydrolyzed casein based diet with the gliadin specific sphingolipid enriched extract added to it.
8. There were significant differences ($P \leq 0.05$) when groups were marked by different letters.

In addition we also studied the pancreatic lymphoid tissue content in BBdp rats fed 5 different diets. The results are listed in Table 5. There were no significant differences observed in pancreatic adipose tissue content in BBdp rats from 5 different dietary treatment groups.

Discussion

In the histopathological analysis, we conducted an assessment of 2 variables, the insulinitis score and the lymphoid tissue content. The findings of the histopathological analysis of the pancreatic tissue specimens of BBdp rats from 5 different dietary treatment groups showed significantly accelerated insulinitis in BBdp rats from the NTP 2000; WG dietary treatment group as compared to the BBdp rats from HC; HCGSF dietary treatment groups. These results correspond to the higher diabetes incidence in BBdp rats fed NTP 2000; WG diet groups as compared to the BBdp rats on HC; HCSF dietary treatment groups (Shi, 2003-M.S. Thesis, Virginia Tech). These results were also in line with the earlier findings where the hydrolyzed casein diet was associated with remarkably lower insulinitis as compared to the wheat flour diet (Hoorfar et al., 1993). Scott et al. (1997) observed a significantly higher insulinitis score in BBdp rats fed NIH diet, a CBD as compared to the hydrolyzed casein diet (Scott et al., 1997), our results demonstrated the same with significantly a higher insulinitis score in NTP 2000 diet, a CBD as compared to the hydrolyzed casein diet, HC. In addition, the hydrolyzed casein diet was reported to be capable of preventing mononuclear cell infiltration in pancreatic islets, resulting in an insulinitis score half of the insulinitis score due to NIH diet, a CBD (reviewed by Scott, 1996). We used NTP 2000 as a CBD, which produced almost 1.5 times the insulinitis score of the HC diet.

When insulinitis scores in BBdp rat fed HC diet vs BBdp rats fed HCGSL diet were compared, a very slight decrease in the insulinitis scores in BBdp rats from

HCGSL diet was observed as compared to the rats from HC dietary treatment groups. These findings, though against our expectation, could be explained as the ability of hydrolyzed casein and lipid from HCGSL diet to prevent mononuclear cell activation, migration and infiltration in pancreatic islet (reviewed by Scott, 1996 and reviewed by Calder, 1998).

However our experimental findings are inconsistent with the findings of Funda et al. (1999). They observed severe terminal stage insulinitis in diabetic animals irrespective of dietary treatments viz. gluten free modified Altromin 1434 diet or standard Altromin 1434 diet. They conducted the feeding trial for 320 days (Funda et al., 1999). The lack of terminal stage insulinitis in our experiment might be due to the fact that the pancreatic tissues from BBdp rats on the different dietary treatments were collected between 63-125 days of the study depending on the diabetes onset. However, by the end of study (125th day), about half of the rats were non-diabetic and the rats those become diabetes fall into recently diagnosed type 1 diabetes category (Shi, 2003-M.S. Thesis, Virginia Tech). It is therefore obvious that terminal stage insulinitis have never attained by almost all of the diabetic BBdp rats in the experiment due to the comparatively short length of the study.

Elliot and Martin (1984), observed normal islets in nondiabetic BB rats by the end of a 133 days feeding trial. Elliott et al. (1988) also observed most of the islets of non-diabetes NOD mice were without insulinitis irrespective of dietary treatments (Elliott et al., 1988). In our study, however all the BBdp rats were found to be associated with islet infiltration irrespective of the autoimmune

diabetes development. There are 3 possible explanations for this. First, a BBdp rat model was used in the Shi (2003) study (M.S. Thesis) instead of the NOD mice in the study conducted by Elliott et al., (1988). Insulinitis in BBdp rats is different than in NOD mice model (reviewed by Mordes et al., 2004). Second, the dietary constituents in the studies by Elliot and Martins (1984) and Elliott et al. (1988) were different from that of Shi's, 2003 study (M.S.Thesis). Third and most important, the time period of the feeding study was only 125 days, though more than 48 % of BBdp rats did not develop overt diabetes (Shi, 2003-M.S. Thesis, Virginia Tech), inflammatory infiltrate of mononuclear cells might have started in their pancreata leading to frank insulinitis (reviewed by Mordes et al., 2004) as we observed in our study. There is scientific evidence that diabetogenic CBD or a diabetes protective hydrolyzed casein diet is capable of inducing insulinitis in non-diabetic animals (Scott et al., 1997)

Interestingly, the islets were without distorted morphology. This might be because the rats were associated with a recent onset or overt diabetes stage in our study. Shrunken and distorted islets are profoundly found in long standing diabetes animals (reviewed by Mordes et al., 2004).

Although no significant differences in the lymphocyte tissue content were found in pancreatic tissues of BBdp rats between any of two diet treatments, the HC diet was found to be related to the least amount of lymphoid tissue aggregates whereas the HCGSF diet fed rats had the highest lymphoid tissue aggregates in their pancreata. It is apparent that the gliadin fraction of CM

soluble sphingolipid enriched extract was responsible for greater lymphoid tissue aggregate formation in the pancreas.

Pancreatic lymphoid tissues have architecture similar to lymph nodes and mainly consist of B cells, T cells, macrophages and DC, indicating their fully immune functionality (Korpershoek et al., 2004). Lymphoid tissues formed in the pancreatic parenchyma play a critical role in the induction and the maintenance of type 1 diabetes as shown in DC-immunized C57BL/6 mice (Ludewig et al., 1998). In addition to this, lymphoid tissue aggregates are present in healthy fetal pancreata (Korpershoek et al., 2004) and in pancreata of diabetic animals (Ludewig et al., 1998), thus whether there is disease or healthy stage does not affect the lymphoid tissue content. Similar way, we observed the presence of pancreatic lymphoid aggregates irrespective of diabetes development in BBdp rats.

Almost all rats have adipose tissues present in their pancreata. No significant differences in adipose tissue content were observed among the 5 dietary treatments however, the NTP 2000 diet was associated with the least adipose tissue content. The NTP 2000 diet has been related to the highest diabetic incidence (Shi et al., 2003-M.S. Thesis, Virginia Tech). This diet might have resulted in highest insulin deficiency leading lipolysis in adipose tissue because insulin deficiency is related to lipolysis in adipose tissues (reviewed by Delaney et al., 2000).

Adipose tissue content and the adipocyte cell size in specific organs such as in the peri-renal part have been shown to be affected by dietary fat (Cha and

Jones, 1998). Another line of evidence showed that dietary lipids are associated with an increase in adiposity (adipocyte mean diameter and volume) in subcutaneous and intramuscular adipose tissues (Gilbert et al., 2003). Thus, the increase in pancreatic adipose tissue content in the HCGSL diet as compared to the HC diet might be due to the sphingolipid fraction of that diet.

We found adipose tissue contained adipocytes with a large central lipid droplet with a very narrow rim around it which indicates that rats from different dietary treatments were well fed because adipocytes accumulate fatty acids after meals, giving large round droplet shapes (unilocular) whereas they get shrunken and acquire elongated shapes during fasting (Cinti, 2005).

Considering adipocytes as an indicator of being well fed, we expected the least content of pancreatic adipose tissues in the WGGSLF diet fed group because the rats of this group gained much less weight, and they refused to eat the diet at the beginning of the Shi, 2003 study (Shi, 2003-M.S. Thesis, Virginia Tech). Our results, however, were inconsistent with our expectations. The reason may be that after making the diet palatable, the rats started gaining weight, and by end of the study, the weight gain of rats from the WGGSLF diet group was about the same as those of the NTP 2000 diet group (Shi, 2003-M.S. Thesis, Virginia Tech), this may result in more adipose tissue content at a later stage.

In summary, the results of histopathological analysis of pancreata of BBdp rats suggest a positive correlation between insulinitis score and diabetes incidence in BBdp rats fed NTP 2000; WG; HC and HCGSL diets individually. In addition,

the findings also suggest that GSLEE is capable of enhancing lymphoid tissue in the pancreata of BBdp rats. Finally the results also suggested an insulinitis induction potential for GSLEE that is possibly due to gliadin's presence in GSLEE.

Chapter 5. Change in Jejunal Histopathology in Type 1 Diabetes : Role of Chloroform Methanol Soluble Gliadin Specific Sphingolipid Enriched Extract.

Abstract

To test whether jejunal histopathological changes occurred due to different dietary treatments during type 1 diabetes pathogenesis, histopathological and immunohistochemical analysis were conducted on jejunal tissue specimens of BBdp rats fed five different dietary treatments. Significantly higher jejunal infiltration score was observed in HC dietary treatment group's rats when compared to the HCGSL dietary treatment group's rats. However there were no significant differences observed in jejunal infiltration score when rats from dietary treatments- NTP 2000, WG and WGGSLF were compared. We did not find significant differences in jejunal mucosal thickness, epithelial erosion and jejunal villi height score when any two dietary treatment groups were compared.

There were no significant differences observed in jejunal CD4+ and $\gamma\delta$ TCR+ cell count / average in 5 fields at 400X between any two dietary treatment groups. However WG diet resulted in the highest jejunal CD4+ cell count and HC diet resulted in the highest small intestinal $\gamma\delta$ TCR+ cell count, although these increases were not significant when compared to other dietary treatments. Insignificant decrease in jejunal CD4+ cell count and $\gamma\delta$ TCR+ cell count were observed in BBdp rats from HCGSL dietary treatment when compared to HC dietary treatment. Similarly, an insignificant decrease in jejunal CD4+ cell count and $\gamma\delta$ TCR+ cell count was observed in WGGSLF dietary treatment group when

compared to WG dietary treatment groups. These results suggest that gliadin specific sphingolipid enriched extract is capable of changing jejunal histopathology.

Introduction

It has been well acknowledged that dysfunction; dysregulation and anomalies of the gut immune system play a critical role in type 1 diabetes development, especially when dietary proteins are the triggers (reviewed by Malaisse et al., 2004). In addition to this, defects in gut immune system have been also suggested in IDDM. For instance Todd et al. (2004), have shown that gut IELs' natural killer (NK) cell deficiencies in number and function are present before disease onset, making BB rats susceptible to development of autoimmune diabetes. These authors further suggested that defects in the gut immune system predispose the animal to systemic autoimmunity (Todd et al., 2004). Further, modulation of the IDDM incidence has been successfully achieved by targeting the gut immune system as an intervention strategy (Scott et al., 2002 and Bellmann et al., 1997).

A wheat based diet is associated with higher disease incidence (60% diabetes incidence) as compared to hydrolyzed casein diet (22% disease incidence) (Hoorfar et al., 1993). A wheat based diet exerts its diabetogenic effect primarily by elevating IFN- γ and TNF- α specific mRNA expression in the small intestine (Flohe et al., 2003). In addition, it enhances proliferation of MLN specific CD3 (+)CD4(+) IFN- γ + T cells whereas suppressing the proliferation of CD4+CD25+ regulatory cells, thus producing Th₁ cytokine bias (Chakir et al., 2005). Importantly, wheat proteins, when fed to NOD mice, have shown to induce type 1 diabetes with an induction of small intestinal enteropathy (Maurano et al., 2005).

Recent studies have attempted to elucidate the potential role of gliadin proteins in inducing type 1 diabetes by activating the gut immune system. Auricchio et al. (2004), showed gut mucosal immune responses (cellular) to gliadin in type 1 diabetes patients in an *in vitro* study (Auricchio et al., 2004). Five out of 19 IDDM patients showed rectal LP and epithelium CD3 and $\gamma\delta$ lymphocytes increments (above the mean + 1 SD) after rectal challenge with gliadins (Troncone et al., 2003). However, none of the studies have fed pure gliadin or gliadin in association with another dietary constituent to assess the jejunal histopathology induction potential of dietary fed gliadin in type 1 diabetes.

Although Shi, 2003 (M.S. Thesis), showed higher diabetes incidence in the WG dietary group as compared to the HC dietary group. However they failed to observe an anticipated high disease incidence in rats from the HCSL diet as compared to the HC diet due to the presence of the CM soluble sphingolipid enriched extract. We have shown evidence for the presence of gliadin proteins in the sphingolipid enriched extract which may have been responsible for the small increase in diabetes incidence in the HCSL group as compared to the HC diet in the Shi, 2003 study (M.S. Thesis).

Here we hypothesized that the CM soluble gliadin specific sphingolipid enriched extract (GSLEE) induces type 1 diabetes by changing jejunal histopathology. To test our hypothesis, we determined jejunal tissue histopathology and the number of CD4+ and $\gamma\delta$ TCR+ cells in the jejunum of BBdp rats fed 5 different diets.

Material and Methods

Materials

Table 6. Total number of H&E stained jejunal tissue slides and frozen jejunal tissue specimens of BBdp rats from 5 different dietary treatment groups.

Abbreviations for the dietary feeding group	Diet	Number of BBdp rats with H&E stained jejunal specimen slides	Number of BBdp rats with jejunal tissue specimen
A	HC	16	15
B	NTP2000	5	5
C	WG	9	8
D	WGGSLF	9	7
E	HCGSL	13	11
		Grand total = 52	Grand total = 46

1. HC-Hydrolyzed casein based diet, which is the negative control.
2. NTP 2000-National toxicology program 2000 diet, which the positive control.
3. WG- Wheat gluten based diet.
4. WGGSLF- Wheat gluten based with the gliadin specific sphingolipid enriched extract removed from the diet.
5. HCGSL- Hydrolyzed casein based diet with the gliadin specific sphingolipid enriched extract added to it.
6. A, B, C, D, E – Abbreviations used for the respective dietary treatments.

H&E stained jejunal tissue slides and jejunal tissue specimens of the BBdp rats from five different dietary treatment groups from the Shi, 2003 study (M.S.

Thesis) were obtained from Dr. Scott's Laboratory, Ottawa Health Research Institute, Ottawa Hospital, General Campus, Canada (enlisted in Table 6). The H & E stained slides and jejunal tissues were stored at 4 °C and -80 °C respectively until used for experimental analysis. Healthy, fresh rat spleen tissue specimens and rat small intestine tissue specimens were run as positive controls in CD4 and $\gamma\delta$ TCR immunohistochemistry. These healthy, fresh rat specimens were obtained from Dr. Bassaganya-Riera, Laboratory of Nutritional Immunology & Molecular Nutrition, Human Nutrition, Food and Exercise Department, Virginia Tech.

Chemicals and Reagents for Immunohistochemistry

TBS TFM (tissue freezing medium) (for frozen tissue specimens), harris modified hematoxylin stains (with acetic acid), histological grade methanol, histological grade acetone, hydrogen peroxide 30%, phosphate buffered saline (PBS) 10X solution, histology grade immu-mount (Thermo Shandon) and gold seal cover glasses (Cat # 3323 size 24*60mm) were purchased from Fisher Scientific CO LLC. Tissue tek crayo molds (disposable vinyl specimen molds, Cat # 4557) were bought from Sakura Finetek, USA, Inc. Normal goat serum was purchased from Sigma-Aldrich Inc. (St. Louis, MO,USA) and poly-L-lysine treated slides (Cat # 7799) were ordered from Lab Scientific Inc. Primary antibodies against rat CD4 (Cat # MCA55R), rat $\gamma\delta$ TCR (Cat # MCA1146) and the corresponding secondary antibodies, STREPTAVIDIN-HRP (Cat # STAR5B) ,

DAB chromogen substrate (Cat # BUF021B) and DAB substrate buffer (Cat # BUF022) were provided by Serotec Inc. (Raleigh, NC, USA).

Assessment of jejunal tissue histopathology

H & E stained jejunal tissue slides were observed under a microscope (Nikon, ECLIPSE ϵ 400) for lymphocyte infiltration with lymphocyte / neutrophil prominence and other histopathological features like mucosal thickness, epithelial erosion and jejunal villi height. Jejunal tissue infiltration was scored on the scale of 1 to with 1 \rightarrow 1-33%; 2 \rightarrow 33-66% and 3 \rightarrow 66-100%.

Five fields were observed under 400X and averaged to obtain the final infiltrations score for that particular tissue. Parameters- mucosal thickness, epithelial erosion and jejunal villi height were scored on the scale of 3 with 200X magnifications.

Immunohistochemistry of jejunal tissue specimen for CD4 and $\gamma\delta$ TCR and enumeration of CD4+ and $\gamma\delta$ TCR + cells.

Immunohistochemistry assay protocol was followed with minor modifications to a published method (Hontecillas et al., 2005). First, tissue sections of the jejunal tissue specimens were cut by using a cryostat (MicroM - HM 505 N) at 8 μ m thickness and mounted on the marked poly-L-lysine slides. The tissue slides were then fixed with 100% histology grade acetone (approximately 250 ml). Next these tissue slides were stored at -20 $^{\circ}$ C till used for immunohistochemistry.

For immunohistochemistry, the tissue slides were equilibrated to room temperature for 1 minute and then were incubated with 1X PBS buffer twice for 5 minutes each. Immediately the tissues slides were incubated with blocking buffer (1X PBS with 10% normal goat serum) @ 0.7ml of blocking buffer/slide for 30 minutes. Then the tissues were incubated at room temperature for 4 hours with primary antibody diluted in 1X PBS with 5% normal goat serum @ of 0.7ml of diluted primary antibody / slide. After two washes the endogenous peroxidase activity was eliminated by using 200 ml of methanol with 30% hydrogen peroxide and incubating the slides for 30 minutes. Again the slides were washed 2 times with 1X PBS and incubated with secondary antibody diluted into 1X PBS with 5% normal goat serum @ 0.7ml of diluted secondary antibody /slide for 1 hour. After two washes with 1X PBS, tissues were incubated with STRAPTAVIDIN-HRP for 30 minutes. Then the tissue slides were washed with 1X PBS twice and a substrate was added on the tissue and incubated until color developed. Then tissue slides were rinsed with nanopure water and were counterstained with hematoxylin. Again, the tissue slides were rinsed using nanopure water and then with tap water. Finally immu-mount was added on the tissue section and it was covered with a cover slip (Hontecillas et al., 2005 with few modifications).

For CD4 immunohistochemistry, primary antibody was used at a concentration of 3 µg/ml and a 1/100 dilution of secondary antibody was used. Primary antibody with a concentration of 4 µg/ml was used for γδTCR immunohistochemistry, with 1/45th dilution of secondary antibody. The secondary antibody used for γδ TCR was HRP conjugated.

These immunohistochemical stained slides were examined by using a differential interference contrast (DIC) microscope (Olympus BX50) attached with digital camera and personal computer equipped with DP Controller image analysis software. CD4 and $\gamma\delta$ TCR positive cells were counted at 400X magnifications (10X ocular and 40X objective lens). Out of two immunohistochemical stained sections on the slide, better immunohistochemical stained tissue section with good histological features was selected. Then 5 microscopic fields of this selected tissue section were randomly chosen and observed under a microscope, and the positive cells/ field were counted and averaged to get the final positive cell count for that particular jejunal tissue specimen.

Statistical Analysis

Histopathology data and data from immunohistochemistry assay were analyzed by one way-ANOVA (analysis of variances) by using SAS software version 9.1 (SAS Institute Inc. Cary, NC, USA). General linear model procedures of SAS were used to perform ANOVA. The differences between means were determined by scheffe's multiple comparisons test. The adjusted means were least square means. Differences were considered significant if $p \leq 0.05$.

The statistical analysis for data of CD4 immunohistochemistry assay was conducted on total of 42 observations because 1 observation from HC group; 2 observations from WG group; 1 observation from WGGSLF group were considered as outliers based on either statistical analysis (Minitab[®] Release 14

.Statistical Software, version 14.20) or the failure of the immunohistochemistry assay on these tissues. In a similar way, the statistical analysis for data of $\gamma\delta$ TCR immunohistochemistry assay was conducted on total of 44 observations as 1 observation from HC group and 1 observation from HCGSL group were considered as outliers because immunohistochemistry assay did not work for these tissues.

Results

Histopathological analysis of jejunal tissue specimens of BBdp rats from five different dietary treatment groups.

Table 7 represents the data of histopathological analysis of jejunal tissue specimens of BBdp rats. The significant increase in the infiltration score of jejunal tissue specimens of BBdp rats fed the HC diet was found as compared to the infiltration score in BBdp rats from WG and the HCGSL dietary treatments. There were no significant differences in infiltration score between any two groups among NTP 2000; WG; WGGSLF and HCGSL dietary treatment groups. Almost all the infiltrated cells in the jejunal mucosa were lymphocytes. For other parameters of the histopathological study of jejunal tissue specimens- mucosal thickness, epithelial erosion and jejunal villi height, there were no significant differences observed when BBdp rats from any two dietary groups among all the five dietary treatment groups were compared.

Table 7. Effect of dietary treatments on the jejunal histopathological parameters.

Dietary treatments	n	Infiltration score	Mucosal thickness	Epithelial erosion	Jejunal villi height
HC	16	1.66 ± 0.07 ^a	1.18 ± 0.09 ^a	1.50 ± 0.14 ^a	1.87 ± 0.16 ^a
NTP 2000	5	1.40 ± 0.13 ^{ab}	1.40 ± 0.16 ^a	2.00 ± 0.26 ^a	1.40 ± 0.28 ^a
WG	9	1.24 ± 0.09 ^b	1.00 ± 0.12 ^a	1.66 ± 0.19 ^a	1.55 ± 0.21 ^a
WGGSLF	9	1.37 ± 0.09 ^{ab}	1.00 ± 0.12 ^a	1.22 ± 0.19 ^a	1.55 ± 0.21 ^a
HCGSL	13	1.27 ± 0.81 ^b	1.30 ± 0.10 ^a	1.53 ± 0.16 ^a	1.76 ± 0.17 ^a

1. All values were reported as LSMEAN ± Std Err LSMEAN and were analyzed by ANOVA by using GLM.
2. n represents the number of BBdp rats in respective dietary treatment.
3. HC represents hydrolyzed casein based diet, which is the negative control.
4. NTP 2000 represents national toxicology program 2000 diet, which is the positive control diet.
5. WG represents wheat gluten based diet.
6. WGGSLF represents wheat gluten based with the gliadin specific sphingolipid enriched extract removed from the diet.
7. HCGSL represents hydrolyzed casein based diet with the gliadin specific sphingolipid enriched extract added to it.
8. There were significant differences ($P \leq 0.05$) when groups were marked by different letters.

Immunohistochemical staining of CD4 cells and analysis CD4+ cell count in jejunal tissue specimen in BBdp rats according to different dietary treatments.

The results of the immunohistochemical stained CD4+ cell count according to different dietary treatment are shown in Table 8. There were no significant differences in jejunal CD4+ cell count/ average in 5 fields at 400X magnifications in BBdp rats between any two dietary treatment groups among

HC; NTP 2000; WG; WGGSLF and HCGSL dietary treatments. However insignificant increase in CD4+cell counts were observed in the BBdp rats from WG diet group when compared to the BBdp rats from WGGSLF dietary treatment group. Similar insignificant increase in jejunal CD4+ cells were observed in BBdp rats from the HC dietary treatment group as compared to the HCGSL dietary treatment group.

Table 8. Effect of dietary treatments on the jejunal CD4+ cell counts / average in 5 fields at 400X magnification.

Dietary treatments	HC (n=15)	NTP 2000 (n=5)	WG (n=8)	WGGSLF (n=7)	HCGSL (n=11)
CD4+ cell count/ average in 5 fields at 400X magnification	106.55 ± 7.51 ^a	108.52 ± 12.57 ^a	114.03 ± 11.47 ^a	90.50 ± 11.47 ^a	79.14 ± 8.47 ^a

1. All values were reported as LSMEAN ± Std Err LSMEAN and were analyzed by ANOVA by using GLM.
2. n represents the number of BBdp rats in respective dietary treatment.
3. HC represents hydrolyzed casein based diet, which is the negative control.
4. NTP 2000 represents national toxicology program 2000 diet, which is the positive control diet.
5. WG represents wheat gluten based diet.
6. WGGSLF represents wheat gluten based with the gliadin specific sphingolipid enriched extract removed from the diet.
7. HCGSL represents hydrolyzed casein based diet with the gliadin specific sphingolipid enriched extract added to it.
8. There were significant differences (P≤0.05) when groups were marked by different letters.

Immunohistochemical analysis of jejuna tissue obtained from BBdp rat showed that CD4+ cells are restricted to the LP as shown in Figure 3. They were

exclusively found as isolated cells. Their pattern was almost the same in jejuna of BBdp rats from 5 different dietary treatments.

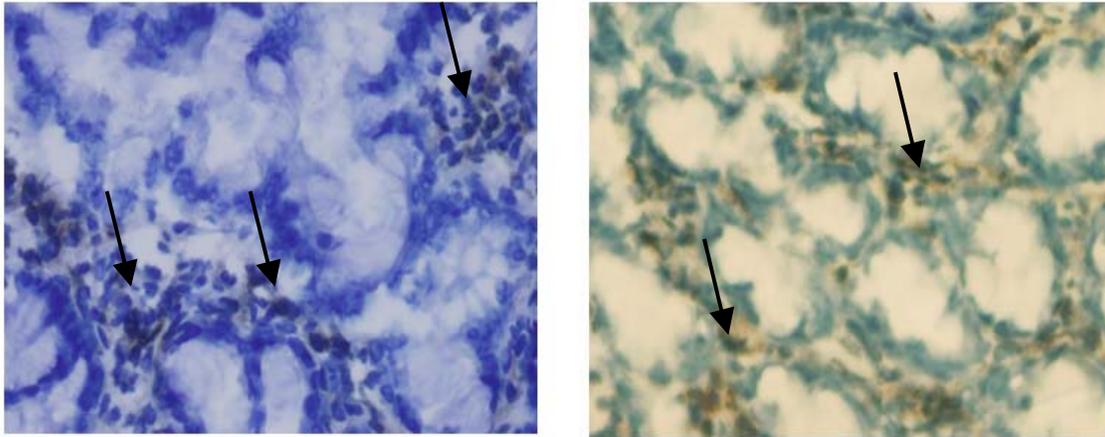


Figure 3. Immunohistochemical staining of jejunal tissue sections from BBdp rat with anti-CD4. Jejunal CD4+ cells were scattered throughout the lamina propria only. Original magnifications 400X and 200X respectively.

Immunohistochemical staining of $\gamma\delta$ TCR cells and analysis $\gamma\delta$ TCR+cell count in jejunal tissue specimen of BBdp rats according to different dietary treatments.

Different dietary treatments influenced the $\gamma\delta$ TCR+ cell counts/average in 5 fields at 400X magnification in jejunal tissue specimens of BBdp rats as presented in Table 9. Although no significant differences were found in jejunal $\gamma\delta$ TCR+ cell counts in BBdp rats from five dietary treatment groups, a trend toward significance in $\gamma\delta$ TCR+ cell counts was observed between the HC diet and the WGGSLF diet group's rats. In addition, an insignificant increase in $\gamma\delta$ TCR+ cell counts in jejunal tissue specimen of BBdp rats were observed in the

HC and HCGSL diet treatment groups as compared to the NTP 2000; WG and WGGSLF dietary treatment groups.

Table 9. Effect of dietary treatments on small jejunal $\gamma\delta$ TCR+ cell counts/ average in 5 fields at 400X magnification.

Dietary treatments	HC (n=15)	NTP 2000 (n=5)	WG (n=8)	WGGSLF (n=7)	HCGSL (n=10)
$\gamma\delta$ TCR+ cell counts/ average in 5 fields at 400X magnification	52.34 \pm 2.24 ^a	47.92 \pm 3.76 ^a	44.70 \pm 2.97 ^a	41.02 \pm 3.17 ^a	49.62 \pm 2.65 ^a

1. All values were reported as LSMEAN \pm Std Err LSMEAN and were analyzed by ANOVA by using GLM.
2. n represents the number of BBdp rats in respective dietary treatment.
3. HC represents hydrolyzed casein based diet, which is the negative control.
4. NTP 2000 represents national toxicology program 2000 diet, which is the positive control diet.
5. WG represents wheat gluten based diet.
6. WGGSLF represents wheat gluten based with the gliadin specific sphingolipid enriched extract removed from the diet.
7. HCGSL represents hydrolyzed casein based diet with the gliadin specific sphingolipid enriched extract added to it.
8. There were significant differences ($P \leq 0.05$) when groups were marked by different letters.

$\gamma\delta$ TCR+cells' were found to be distributed in the LP and epithelial regions as well. They were found as isolated cells and in clusters as shown in Figure 4. Their distribution pattern did not vary with the dietary treatments.

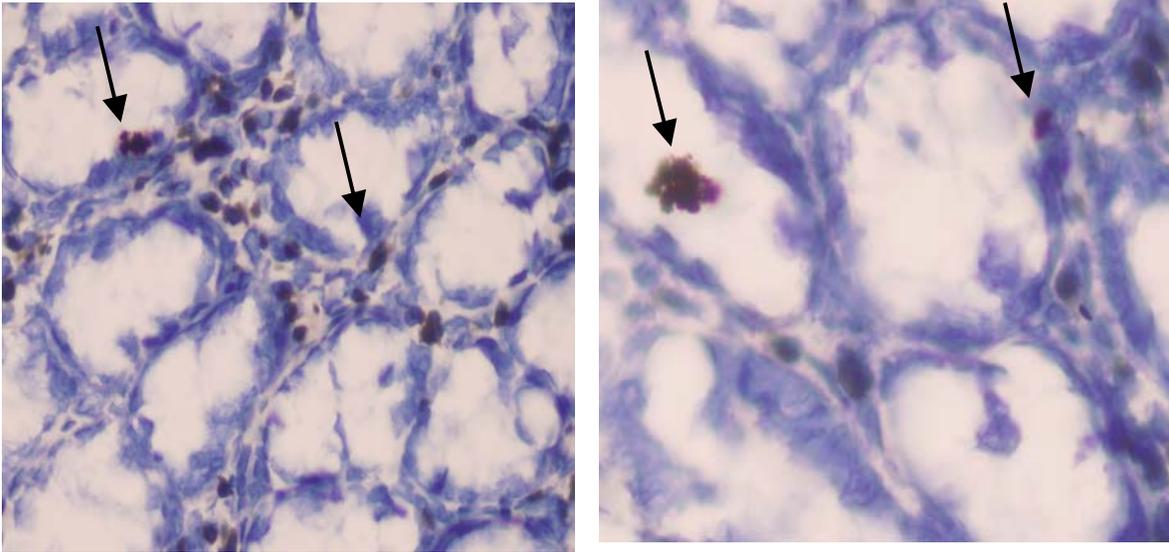


Figure 4. Immunohistochemical staining of jejunal tissue sections of BBdp rat with anti- $\gamma\delta$ TCR. $\gamma\delta$ TCR+ cells are scattered throughout the lamina propria and epithelium. Original magnifications 400X.

Discussion

Histopathological analysis of jejunal tissue of BBdp rats from 5 different dietary treatments.

H & E stained jejunal tissue sections were evaluated for histopathological changes. Histopathological manifestation of jejunal tissue of BBdp rats includes an increase in infiltration score, increase in mucosal thickness, increase in epithelial erosion and completely flattened jejunal villi.

We hypothesized that the CM soluble GSLEE possesses potential to cause inflammation of the jejunum in type 1 diabetes, however, we failed to observe the corresponding higher infiltration score and jejunal villi flattening in BBdp rats from the HCGSF diet as compared to the BBdp rats fed HC diet. In contrast, we observed significant increase in the jejunal infiltration score in BBdp rats of the HC diet group as compared to the BBdp rats of HCGSF group. This could be because of the anti-proliferative potential of the sphingolipid breakdown products (reviewed by Hannun and Linares, 1993), which impact activation, proliferation and differentiation, the main stages during activation of T lymphocyte after antigenic stimuli (reviewed by Martinova, 1998). Similarly, the HC group had a significantly higher infiltration score as compared to the WG diet which may be due to the migration of the most of the WG activated intestinal mucosa lymphocytes into the pancreatic lesion leading to the disease pathogenesis, leaving less inflammatory cells in the small intestine. Although a HC diet is a protective diet in animal model of type 1 diabetes, the highest jejunal infiltration score associated with this diet could be attributed to the highest number of immunoregulatory $\gamma\delta$ TCR⁺ cells observed in jejunum of BBdp rats from this diet as shown by immunohistochemistry assay.

Findings of an earlier study demonstrated that the NTP 2000 diet is associated with a higher intestinal permeability index when compared to the hydrolyzed casein diet as observed in BBdp rats (Courtois et al., 2005), which results in the altered and aberrant mucosal immunity to the dietary antigens (reviewed by Vaarala, 1999). Considering these findings and diabetes incidence

due to NTP 2000 diet and the HC diet in Shi's 2003 study (Shi, 2003-M.S. Thesis, Virginia Tech), we predicted that the NTP 2000 diet, our positive control group, would have the highest jejunal histopathology and intestinal enteropathy. However, the NTP 2000 diet was found to be associated with less infiltration of inflammatory cells and jejunal villi height in BBdp rats when compared to the HC dietary treatment. Conversely, BBdp rats from the NTP 2000 diet had a higher epithelial erosion and mucosal thickness than the BBdp rats of the HC diet. Thus our results are consistent with our prediction for two parameters and inconsistent for the remaining two parameters of the jejunal histopathology study.

The WG dietary group was found to be associated with the lowest inflammatory cell infiltrate, mucosal thickness and jejunal villi height score in BBdp rats among all the dietary treatment groups. In spite of this, the highest CD4+ positive cell count was found in the WG dietary treatment group. However, there may be a possibility of the presence of intestinal enteropathy even in a morphological normal intestine, as was observed by Raia et al. (2000) in intestine of cystic fibrosis condition (Raia et al., 2000).

Our findings regarding insignificant differences in the jejunal villi flattenings among the BBdp rats from NTP 2000; WG and HC dietary treatments are in agreement with the previous studies conducted with similar diet types and BBdp rats (Graham et al., 2004). Similar to our findings, Graham et al. (2004) did not find significant differences in villus shortening in the BBdp rats fed diabetes promoting diet (WG and NTP 2000) or diabetes protective diet (hydrolyzed casein diet) (Graham et al., 2004).

In regards to mucosal thickness and epithelial erosion, we found the highest score in the NTP 2000 diet and the second highest in the WG group, which is consistent with respective higher diabetes incidence in BBdp rats from these two dietary treatment groups in Shi's, 2003 study (M.S. Thesis). However, the differences were not significant among the 5 different dietary treatments when these two parameters are considered. An increase in mucosal thickness we observed may be a result of pronounced crypt elongation and hyperplasia of crypt epithelial cells as observed in the Graham et al. (2004) study (Graham et al., 2004).

At the time of dissecting the BBdp rats, morphological findings include an inflamed gut in rats with type 1 diabetes whereas rubbery guts occurred in rats without type 1 diabetes (Shi, 2003-M.S Thesis-Virginia Tech). Despite these observations, the jejunal tissues of all rats have manifested infiltration irrespective of diabetes development, possibly because physiological inflammation is the characteristic feature of a healthy small intestine (reviewed by Mayer, 2000).

Jejunal CD4+ and $\gamma\delta$ TCR + cell count/ average in 5 fields at 400X magnification among different dietary treatment by immunohistochemical staining.

Our study found the highest number of jejunal CD4+ positive cells in the WG dietary treatment group's BBdp rats, however, the increase was not significant. These findings strengthen the earlier finding about wheat gluten's

potential to induce T cell responses (reviewed by Akerblom and Knip, 1998). An insignificant increase in CD4+ T cell count was observed in the WG diet group rats when compared to the WGGSLF diet group rats, which implies gliadin from the CM soluble sphingolipid enriched extract resulted in enhanced infiltration of the CD4+ cells into intestinal mucosa as observed by Stepankova et al. (2003) (Stepankova et al., 2003). When these fractions were removed from the chloroform methanol extract, it resulted in a decrease in intestinal CD4+ cell number. The fact that gliadins (peptic-tryptic digest) can activate intestinal CD4+ cells and accumulation of these activated CD4+ cells takes place in LP is well established in celiac disease, which is an autoimmune disease and is associated with type 1 diabetes (Maiuri et al., 1996). Gliadin could also activate peripheral lymphocytes and extravasation of these circulating lymphocytes in the pancreas islet could be possible resulting in type 1 diabetes development (reviewed by Vaarala, 2000). These types of immune responses may become autoaggressive due to genetic predisposition or local cytokine environment leading to the destruction of β cells.

Our findings may help to explain the results of disease incidence in the Shi, 2003 study (M.S. Thesis), the first part of our study. They found higher type 1 diabetes incidence in the WG group rats as compared to the HCSL group rats at 125 days, which is consistent with the intestinal CD4+cell count in the jejunal tissues of BBdp rats of respective dietary treatment groups. However, the NTP 2000 diet, which proved to be the most diabetogenic diet at 125 days with 78 % disease incidence (Shi, 2003-M.S. Thesis, Virginia Tech), was observed to have

the second highest CD4⁺ cell count. Although this diet was associated with fewer jejunal CD4⁺ cells as compared to the WG diet, there is a possibility that the majority of these CD4⁺ cells were CD3⁺CD4⁺IFN- γ ⁺ i.e. Th₁ type as observed by Chakir et al (2005) in their study (Chakir et al., 2005). In addition, it is also possible that the extravasations of NTP 2000 diet activated lymphocytes in the pancreatic lesion has occurred (Lohmann et al., 2002), resulting in lower count in small intestine and higher diabetes incidence in BBdp rats.

Although the HC diet was the least diabetogenic one and was the negative control in the Shi's, 2003 study (M.S. Thesis), it has a greater jejunal CD4⁺ cell count than the HCGSF and WGGSLF dietary treatment groups. Further the HCGSL diet was found to be associated with the lowest CD4⁺ cell counts among all the dietary treatment groups, which were against our expectation. Both of these findings could be explained as the sphingolipid from HCGSL diet may have counter affected the CD4⁺ activation potential of the gliadin from GSLEE since the sphingolipid breakdown products have documented to act on anti-proliferative pathways of cell regulation (reviewed by Hannun and Linardic, 1993). Secondly, the fact about the presence of an extreme small quantity of gliadin in the HCGSL diet may be accounted for less immune cell activation.

Graham et al. (2004) have found no significant differences in jejunal CD4⁺ T cells in BBdp rats fed NTP 2000 and the HC diets (Graham et al., 2004). We did not find significant differences in jejunal CD4⁺ cell count in BBdp rats fed either NTP 2000 or HC diets.

The exclusive distribution of CD4⁺ cells in the LP is consistent with the result of the Graham et al. (2004), study. They found CD4⁺ cells in the LP of BBdp control rats and BBdp rats irrespective of the diabetes promoting or diabetes protecting diets fed to them (Graham et al., 2004).

When jejunal $\gamma\delta$ TCR⁺ cell count among 5 different dietary treatment groups are considered no differences were significant. A marked though insignificant increase in $\gamma\delta$ TCR⁺ cells was observed in jejunal tissue of BBdp rats from HC and HCGSF diets. These were the two diet groups that were associated with the least diabetes incidence in the Shi, 2003 study (M.S. Thesis). In contrast, a decrease in jejunal $\gamma\delta$ TCR⁺ cell count in BBdp rats of NTP 2000; WG and WGGSLF diet groups has been observed though these groups have demonstrated the higher diabetes incidence in Shi's, 2003 study (Shi, 2003-M.S. Thesis). These findings emphasize the immunoregulatory role of $\gamma\delta$ T cells, which are believed to be the first line of defense at intestinal mucosal system (reviewed by Hayday and Tigelaar, 2003). It is apparent that the increase in $\gamma\delta$ TCR⁺ cells contributed to the reduction of the intestinal inflammation and amelioration of the disease incidence as discussed (reviewed by Hayday and Tigelaar, 2003).

As postulated, gliadins possess the potential to activate $\gamma\delta$ TCR cells. Challenging rectal biopsy specimen of IDDM patients with frazer's peptic-tryptic digest of gliadins resulted in increase in small intestine $\gamma\delta$ TCR positive cell count mean + 1 SD in 25% of IDDM patient as compared to the control subjects (Troncone et al., 2003). Thus the lower count of jejunal $\gamma\delta$ TCR⁺ cells in BBdp

rats from the WGGSLF dietary group may be accountable to lack of α and γ gliadins in this diet.

$\gamma\delta$ TCR iIELs express KGF that is responsible for tissue repair and epithelial regeneration as shown in the dextran sodium sulfate (DSS) induced mouse colitis model system (Chen et al., 2002). Thus an increase in $\gamma\delta$ TCR+ cells would result in less epithelial erosion. Our findings also demonstrated this. There were higher $\gamma\delta$ TCR + cell counts in the BBdp rats fed HC and HCGSF diets than in the rats from NTP 2000 and the WG diet groups and respective low jejunal epithelial erosion in BBdp rats fed the HC and HCGSF diet as compared to the NTP 2000 and WG dietary treatment groups.

Distribution of $\gamma\delta$ TCR+ cells in the epithelium and LP compartments is consistent with the findings obtained by Savilahti et al., (1999) where the investigators observed $\gamma\delta$ TCR+ cells distributed in the epithelium and LP of jejunum of patients with type 1 diabetes as well as in human controls (Savilahti et al., 1999).

When jejunal CD4+ cells and $\gamma\delta$ TCR+ cells were compared, the average number of jejunal CD4+ cells per field was greater than the average number of jejunal $\gamma\delta$ TCR+ cells per field. Savilahti et al., (1999) observed a similar trend in the jejunum of type 1 diabetes patients (Savilahti et al., 1999). However, no correlation was found between CD4+ and corresponding $\gamma\delta$ TCR + counts in the jejunum of BBdp rats fed 5 different diets. When individual animal and average CD4+ and $\gamma\delta$ TCR + cells are compared, no correlation between diabetes incidence and respective cell positive count was found. Many times non-diabetic

BBdp rats were found to be associated with higher positive cell counts. This could be because intestinal enteropathy with increase in CD4+ and CD8+ cells is a consistent feature of the intestinal mucosa of BBdp rats (Graham et al., 2004).

Alternatively, the cell counts might have changed with the disease development. In humans, decreased density of CD4+ cells have been found in IDDM diabetes patient when compared to the human controls (Savilahti et al., 1999). Increases in peripheral $\gamma\delta$ TCR cells have been also found at the onset of diabetes (Funda et al., 1995). Variation in $\gamma\delta$ T cell count depending on age and the stage of the type 1 diabetes development has been demonstrated as well (Funda et al., 1995).

In the present study, the immunohistochemical staining for CD4+ and $\gamma\delta$ TCR + cells was been conducted on tissue specimens collected between 63-125 days of the experimental feeding period (125 days). Thus this study only considered one time-point during disease development; however, cell responses might have peaked immediately after the CM soluble gliadin presentation to the gut immune cells.

Our experimental results showed prevalence of both CD4+, $\gamma\delta$ TCR+ cells in the jejunum of BBdp rats with type 1 diabetes which is similar to the prevalence of these cell populations in jejunum of humans suffering from type 1 diabetes (Savilahti et al., 1999). The CD4+ cell count and the $\gamma\delta$ TCR + cell count were not correlated with histopathology parameters except that an increase in infiltration score was associated with corresponding increase in the CD4+ cell count. As both WG and WGGSLF diets resulted in about the same score for

histopathological analysis of jejunal tissue and jejunal $\gamma\delta$ TCR+ cells. There is possibility that glutenin fractions, a diabetogen (reviewed by Scott, 1996) is present in the residue which might have activated and have proliferated the gut immune cells.

In conclusion, the findings of the histopathological and immunohistochemical analysis of jejunal tissue specimens suggest that NTP 2000 and WG diets induced type 1 diabetes in BBdp rats by changing the jejunal histopathology. Although the results are not clear enough to draw the conclusion that gliadin from GSLEE changed jejunal histopathology as anticipated, the findings are suggestive of suppressor potential of sphingolipid on jejunal histopathology.

Chapter 6. Serum IFN- γ and TNF- α Concentrations Change in Type 1 diabetes : Role of Chloroform Methanol Soluble Gliadin Specific Sphingolipid Enriched Extract.

Abstract

A Th₁ cytokine bias has been reported in type 1 diabetes. Therefore to test whether dietary treatment changes the Th₁ and proinflammatory serum cytokine levels in association of type 1 diabetes, we conducted a sandwich ELISA assay on the serum sample from BBdp rats from five different dietary treatment groups. Significantly higher serum IFN- γ concentrations were found in WGGSLF dietary treatment groups BBdp rat when compared to the rest of the dietary treatment group's rats. There was a significant increase in serum IFN- γ concentrations in BBdp rats from HCGSL dietary treatment group when compared to HC dietary treatment group's rats. However no significant difference in serum IFN- γ concentrations was found when BBdp rats from NTP 2000, WG and HCGSL dietary treatment groups were compared. For serum TNF- α concentrations, HC diet was found to be associated with highest serum TNF- α concentrations when compared to rest of the dietary treatment, although this increase was not significant. There were no significant differences observed in serum TNF- α concentrations between any two dietary treatment groups. Thus we demonstrated that gliadin specific sphingolipid enriched extract changes serum IFN- γ and TNF- α concentrations in type 1 diabetes.

Introduction

Diet has a significant influence on the serum IFN- γ and TNF- α levels in autoimmune diseases and in infections. Dietary fish oil contains ω -3 PUFAs, which are able to reduce the serum IFN- γ levels significantly in mice challenged with *Listeria monocytogenes* (Fritsche et al., 1999). Common allergenic foods were shown to increase serum TNF- α level significantly in prick-positive patients with rheumatoid arthritis, an autoimmune disease (Karatay et al., 2004). Serum IFN- γ levels and TNF- α levels have also been correlated with disease pathogenesis in type 1 diabetes (Schloot et al., 2002 and Cavallo et al., 1991). *In vitro* stimulation of PBMC by BLG from cow milk protein enhanced the IFN- γ synthesis both in type 1 diabetes patients and controls as well (Karisson et al., 2001). Gliadin is capable of stimulating IFN- γ production in culture supernatants of PBMCs (Peripheral blood mononuclear cells) (O'keeffe et al., 1999) or jejunal T cell clones in coeliac disease patients (Troncone et al., 1998), an autoimmune disease related to IDDM.

Almost all of these studies emphasized either on the effects of type 1 diabetes development (diabetics vs nondiabetics or recently diagnosed type 1 diabetes patients vs long standing type 1 diabetes patients) on serum Th₁ and proinflammatory cytokine levels (Cavallo et al., 1991) or the effects of immunotherapies i.e. cyclosporine A on the serum cytokine concentrations (Burke et al., 1994). Also, some studies demonstrated how diet modulates the cytokine expression in the pancreas, small intestine and PBMC (Scott et al., 1997, Flohe et al., 2003 and Karisson et al., 2001). However, there is no

published study linking the influence of diabetogenic or diabetes protective diets on endogenous serum IFN- γ and TNF- α levels in type 1 diabetes. In this experiment, we determined IFN- γ and TNF- α concentration in sera of BBdp rats from dietary treatment to assess if the dietary treatment affected the Th₁ and proinflammatory serum cytokine concentrations during type 1 diabetes development.

Materials and Methods

Materials

Sera samples from BBdp rats in the five different dietary feeding groups from the Shi, 2003 study (M.S. Thesis) were obtained from Dr. Scott's Laboratory, Ottawa Health Research Institute, Ottawa Hospital-General Campus, Canada. These sera samples were stored at -80 °C until used for ELISA assay. One sample in the WGGSLF group was not enough for both IFN- γ and TNF- α assay, so we used 17 instead of 18 samples of the WGGSLF group for TNF- α assay.

Table 10. Total numbers of sera samples of BBdp rats from 5 different dietary treatment groups.

Abbreviations for the dietary feeding group	Dietary group	Number of BBdp rats with sera samples
A	HC	20
B	NTP 2000	16
C	WG	17
D	WGGSLF	18
E	HCGSL	17
		Grand total = 88

1. HC-Hydrolyzed casein based diet, which is the negative control.
2. NTP 2000-National toxicology program 2000 diet, which is the positive control.
3. WG- Wheat gluten based diet.
4. WGGSLF- Wheat gluten based with the gliadin specific sphingolipid enriched extract removed from the diet.
5. HCGSL- Hydrolyzed casein based diet with the gliadin specific sphingolipid enriched extract added to it.
6. A, B, C, D, E – Abbreviations used for the respective dietary treatments.

Chemicals and Reagents

Rat IFN- γ (Interferon-gamma) ELISA Ready-SET-Go! (Cat # 88-7315) and rat TNF- α (Tumor necrosis factor-alpha) ELISA Ready-SET-Go! (Cat # 88-7340) were purchased from the eBioscience, Inc. (San Diego, CA, California, USA). Tween-20 was obtained from Sigma-Aldrich. Stop solution (2N H₂SO₄) and phosphate buffered saline (PBS-10X) were purchased from Fisher-Scientific Co LLC.

Quantification of IFN- γ and TNF- α concentration in the rat sera by ELISA

IFN- γ and TNF- α levels in the rat sera were quantified by sandwich ELISA as per the manufacturer's recommended protocol (eBioscience, San Diego, CA, California, USA).

A 96 well NUNC Maxisorp flat-bottom plate was coated by either 100 μ l of anti IFN- γ or 100 μ l of anti TNF- α antibodies in 1X coating buffer (0.1M NaHCO₃) and the plate was sealed and incubated overnight at 4 °C. The next day, the wells were aspirated and washed with 300 μ l of washing buffer (0.05% Tween-20 in PBS) followed by inverting and blotting against the absorbent paper to remove any residue. Three consecutive washes (4 minutes/wash) were employed. Then the plate was incubated with 1X assay diluent for 1 hour at room temperature with 200 μ l of assay diluent /well. Again the plate was aspirated and washed twice with ~300 μ l of washing buffer.

Then 100 μ l of standard/well was added to appropriate wells followed by 100 μ l of the sample/well as per the plate template design. To obtain the standard curve, two-fold serial dilutions of the top standard were employed and the plate was incubated for 2 hours. Then the wells were aspirated and washed five times with washing buffer. Appropriate secondary antibody conjugated with biotin, 100 μ l/ well was added and the plate was incubated for 30 minutes at room temperature. Again the wells were aspirated and washed five times with washing buffer. One hundred microliter of avidin-HRP diluted in 1X assay diluent /well was added to each well and the plate was sealed and incubated for 30 minutes at room temperature. Prior to adding the 100 μ l of substrate/well, 7

washes of washing buffer were repeated. After the substrate addition, the plate was incubated for 15 minutes or until the color developed. Finally 50 µl of stop solution was added to each well.

The plate was read with microplate reader µQuant (BIO-TEK Instruments Inc.). Software KC Junior was used to record the absorbances, to generate standard curve and to calculate the corresponding cytokine concentrations in pg/ml. The detection limit of this assay was at picogram (pg) levels, with standard curve ranges 15 pg/ml-2000 pg/ml and 16 pg/ml-2000 pg/ml for IFN-γ and TNF-α respectively.

Statistical Analysis

Data obtained by ELISA experiments were analyzed by one way-ANOVA (analysis of variance), using SAS software version 9.1 (SAS Institute Inc. Cary, NC, USA). General linear model procedures of SAS were used to perform ANOVA. The differences between means were determined by scheffe's multiple comparisons test. The adjusted means were least square means. Differences were considered significant if $p \leq 0.05$.

The statistical analysis for the data of IFN-γ serum concentration was conducted on total of 86 observations because 1 observation from WG group and 1 observation from HCGSL group were considered as outliers based on the statistical analysis (Minitab[®] Release 14 Statistical Software, version 14.20) and storage condition problem. In similar way, 1 observation from HC group, 1 observation from NTP group and 1 observation of WG group were considered as

statistical outliers out of total of 87 observations for TNF- α serum concentration data analysis.

Results

Determination of IFN- γ concentrations in sera samples in BBdp rats from 5 different dietary treatments by using ELISA

A graph presenting IFN- γ concentrations in sera samples of BBdp rats is shown in Figure 5. Significant increase in IFN- γ sera concentrations was found in the WGGSLF diet group's BBdp rats as compared to all other diet groups' BBdp rats- HC; NTP2000; WG and HCGSL. IFN- γ sera concentration in BBdp rats of the HC was found significantly lower than in the BBdp rats of WG; WGGSLF and HCGSL diet groups. Insignificant elevation in the IFN- γ sera level in the NTP 2000 diet group's BBdp rats was observed as compared to the HC diet group's rats. There were no significant differences in the serum IFN- γ levels in BBdp rats from NTP 2000 and WG diet groups. Serum IFN- γ level was not detectable in 30% (26 rats out of total 86) irrespective of diabetes incidence in the Shi, 2003 study (M.S. Thesis). However, most of these rats (16 out of 26) belong to the HC dietary treatment group.

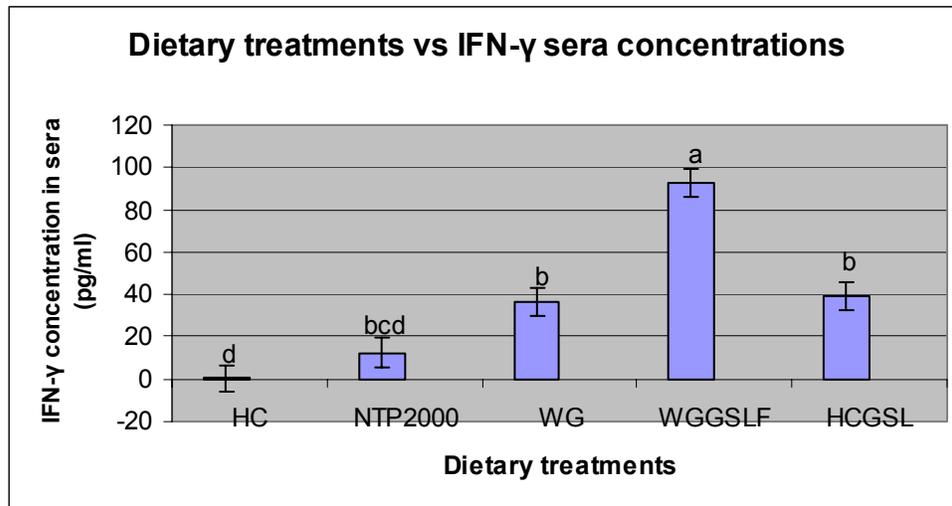


Figure 5. Effect of dietary treatments on serum IFN- γ concentration. Values are LSMEAN with Std Err LSMEAN computed by ANOVA. HC represents hydrolyzed casein based diet, which is the negative control; NTP 2000 represents national toxicology program 2000 diet, which is the positive control; WG represents wheat gluten based diet; WGGSLF represents wheat gluten based with the gliadin specific sphingolipid enriched extract removed from the diet and HCGSL represents Hydrolyzed casein based diet with the gliadin specific sphingolipid enriched extract added to it. Different letters above each Y error bar shows significant differences ($P \leq 0.05$).

Determination of TNF- α concentrations in sera samples of BBdp rats by using ELISA.

The results of ANOVA analysis of TNF- α concentration in rat sera samples are presented in Table 11. The highest sera TNF- α concentrations were found in the rats from HC diet group, but this increase was not significant when compared to other groups. No significant differences were observed in any two groups' rats among all the dietary treatments for sera TNF- α concentration. Insignificant increases in sera TNF- α concentration in the WGGSLF dietary treatment group were observed as compared to the WG dietary treatment group. TNF- α was detectable in 76 of 85 rats (~90%) irrespective of diabetes incidence.

Table 11. Effect of dietary treatments on serum TNF- α concentrations.

Dietary treatments	HC (n=19)	NTP 2000 (n=16)	WG (n=17)	WGGSLF (n=16)	HCGSL (n=17)
TNF-α level (pg/ml)	2.53 \pm 0.32 ^a	1.66 \pm 0.36 ^a	1.50 \pm 0.34 ^a	1.62 \pm 0.35 ^a	1.80 \pm 0.34 ^a

1. All values were reported as LSMEAN \pm Std Err LSMEAN and were analyzed by ANOVA by using GLM.
2. n represents the number of BBdp rats in respective dietary treatment.
3. HC represents hydrolyzed casein based diet, which is the negative control.
4. NTP 2000 represents national toxicology program 2000 diet, which is the positive control diet.
5. WG represents wheat gluten based diet.
6. WGGSLF represents wheat gluten based with the gliadin specific sphingolipid enriched extract removed from the diet.
7. HCGSL represents hydrolyzed casein based diet with the gliadin specific sphingolipid enriched extract added to it.
8. There were significant differences ($P \leq 0.05$) when groups were marked by different letters.

The average concentration of serum IFN- γ was higher than the average concentration of serum TNF- α in BBdp rats from all dietary treatment except those fed the HC diet.

Discussion

Serum IFN- γ concentration in BBdp rats from 5 different dietary treatments

We observed a significant increase in sera IFN- γ concentrations in the WGGSLF group's BBdp rats as compared to the HC; WG; NTP 2000 and HCGSF dietary treatment groups' BBdp rats. Significantly lower serum IFN- γ levels in NTP 2000 and WG diet group's BBdp rats as compared the WGGSLF diet group's BBdp rat could be because of the extravasations of the NTP 2000 / WG diet activated Th₁ lymphocytes to the inflamed pancreas (Lohmann et al., 2002) accounting for lower number of lymphocytes in periphery with low IFN- γ production.

Secondly, the WGGSLF diet was almost devoid of any lipid fraction. As dietary lipid viz. fish oil (rich in *n*-3 PUFAs) possess the potential to suppress serum IFN- γ levels significantly in *Listeria monocytogenes* infection study conducted in BALB/c mice particularly when mice fed a fish oil enriched diet before experimental infection of *Listeria monocytogenes* (Puertollano et al., 2005) and splenic lymphocytes from the mice fed fish oil showed decreased expression of mRNA IFN- γ (Wallace et al., 2001), the absence of lipids in WGGSLF might have indirectly helped to elevate IFN- γ production. Also, it is probable that there is glutenin in residue in the WGGSLF diet, which might have stimulated synthesis of IFN- γ . Glutenin peptides may have activated small intestinal T cells and contributed to disease pathogenesis by secreting large amount of IFN- γ as was observed in celiac disease (van de Wal et al., 1999).

As expected, the HC diet, the protective diet in type 1 diabetes was found to be associated with the least serum IFN- γ levels among all dietary groups. On the other hand, HC diet resulted in highest jejunal $\gamma\delta$ TCR + cells. It is possible that increase in jejunal $\gamma\delta$ TCR + cells, an immunoregulatory T cells may have contributed to less serum IFN- γ levels. $\gamma\delta$ IELs exert suppressive effect on IFN- γ production by IEL as shown by Inagaki-Ohara et al.(2004) (Inagaki-Ohara et al., 2004). Also significant elevation in serum IFN- γ levels in BBdp rats from the HCGSL diet compared to the BBdp rats fed HC diet was found which indicates that gliadin fraction from the sphingolipid enriched extract induces IFN- γ production. Peptic \pm tryptic digest (PT) of gliadin challenged T cell clone lines obtained from celiac disease patients have been observed to synthesize the highest level of IFN- γ cytokine as compared to measurable IL-4 cytokine production (Troncone et al., 1998). There were no significant differences in the serum IFN- γ cytokine levels in BBdp rats from NTP2000 and WG diet groups because both of these diets have wheat protein as a source of protein.

There is experimental evidence that cereal-based diets (wheat + corn) are associated to high IFN- γ mRNA expression in the small intestine of NOD mice (Maurano et al., 2005). Additionally, the CBD, NTP 2000 diet is associated with increased pancreatic IFN- γ expression; conversely, hydrolyzed casein diet suppresses it (Scott et al., 1997). Importantly, serum IFN-gamma levels increase with the increase in IFN- γ mRNA expression in the pancreas between 5 and 14-16 weeks of age (Schloot et al., 2002). These findings give rise to the possibilities that serum cytokine IFN- γ levels especially, for NTP 2000; WG and

HC diet groups that we observed in our experiment, may be due to local production of IFN- γ in the pancreas or gut tissue and transit of these cytokines into the circulation.

We also speculate that the peripheral lymphocytes contribute to serum IFN- γ cytokine levels. At the onset of the IDDM and in a 15 month follow up study, significantly lower IFN- γ specific peripheral T cells (CD3+, CD4+ and CD8+) were observed in IDDM patient as compared to the human controls. The investigator suggested that there was migration of these peripheral lymphocytes to the pancreas at the onset of the disease (Avanzini et al., 2005). Decrease in spontaneous IFN- γ production soon after the diagnosis of the IDDM have been observed in an *in vitro* spontaneous secretion assay after stimulating PBMC with antigens as measured by ELISA (Karlsson Faresjo et al., 2004). Thus lower IFN- γ concentration in NTP 2000 dietary treatment could be due to the fact that most of the rats of this dietary treatment fall under newly diagnosed stage and PBMC from these rats have synthesized less IFN- γ . Secondly, MLN (draining into the gut) lymphocytes of BBdp rats fed the NTP 2000 diet showed a IFN- γ + phenotype upon activation (Chakir et al., 2005). Migration of these lymphocytes to the pancreatic lesion contributing less amounts of IFN- γ toward serum IFN- γ level seems possible as well.

In addition, the second least serum IFN- γ level in NTP 2000 diet group's rats, though highest diabetes incidence due to these diet in Shi, 2003 study (M.S. Thesis) leads to the conclusion that sera cytokine levels may not reflect the cytokine produced by individual cell type upon activation by gliadins from GSLEE

and cytokine responsible for the induction of type 1 diabetes. Vankayalapati et al. (2003), found discordance in serum cytokine levels in tuberculosis patients and *Mycobacterium tuberculosis* induced cytokine production by PBMCs (Vankayalapati et al., 2003).

When individual animal diabetes incidence and corresponding serum IFN- γ levels were compared, serum IFN- γ levels were not detected in some rats particularly in those rats from the HC diet group, which never became diabetic. Also it is notable that only 4 BBdp rats from the HC diet have detectable IFN- γ levels. In the NTP 2000 and WG dietary groups, the rats without diabetes had very low serum IFN- γ levels. In contrast, although the WGGSLF dietary group rats didn't become diabetic, they had markedly increased serum IFN- γ levels. There is a possibility of a presence of wheat protein, glutenin in the residue after the CM extraction. BBdp rats from WGGSLF dietary regimen might have been on the verge of developing the disease due to glutenin (reviewed by Scott et al, 1996), but the limited length of the feeding trail (125 days) made it impossible to record the further diabetes incidence. An increased serum IFN- γ level is an indicator of the prediabetic period or onset of the disease in type 1 diabetes (Schloot et al., 2002). Thus, elevated serum IFN- γ cytokine levels could be an indicator of the prediabetic period in the WGGSLF dietary group's rats.

Serum TNF- α concentration in BBdp rats from different dietary treatments by using ELISA.

BBdp rats from the HC dietary group were found to have higher levels of serum TNF- α concentration than rats from the NTP 2000; WG; WGGSLF and HCGSL dietary treatment groups, however the differences were not significant. Reduction in serum TNF- α levels in the HCGSF group as compared to the HC group may be attributed to the presence of lipids in the CM soluble extract. Puertollano et al.(2005) observed the modulatory role of dietary lipids (olive oil or fish oil) on serum TNF- α levels (Puertollano et al., 2005).

The significant increase in TNF- α mRNA expression in the small intestine is observed due to the wheat based NTP 2000 diet opposed to semi-purified hypoallergenic Prosobee diet fed group and Prosobee + casein diet fed group (Flohe et al., 2003). However, TNF- α expression is not detectable in the pancreata of patients with a recent onset of the disease (Huang et al., 1995). As ~ 78 % of rats from the NTP 2000 group and ~ 50% of rats from the WG and WGGSL groups fall under the recent onset of disease stage (Shi, 2003-M.S. Thesis, Virginia Tech), it is clear that these rats must not have an increase pancreatic TNF- α level.

Thus increased serum TNF- α levels may be the reflection of an increased synthesis of TNF- α by by peritoneal macrophages and circulating peripheral blood lymphocytes or local production in the small intestine (Flohe et al., 2003). Local production in the pancreas as suggested by Cavallo et al. (1991) (Cavallo et al., 1991) seems less likely to be attributed to the serum TNF- α level.

Peritoneal macrophages from BBdp rats (as compared to the diabetes resistant BB rats or Wister rats) usually secrete much more TNF- α upon stimulation, which is due to enhanced gene transcription and translation in BB rats (Rothe et al., 1994). It is quite probable that hydrolyzed casein proteins from the HC diet, though protective, might activate peritoneal macrophages resulting in high TNF- α synthesis, raising serum TNF- α levels, similar to the observation that n-3 PUFA, though suppresses PBMC production of TNF- α (Endres et al., 1989), enhances peritoneal macrophage production of TNF- α significantly (Hardardottir and Kinsella, 1991).

Approximately 40% of BBdp rats from WG diet group fall under prediabetic category. Therefore the least TNF- α level in BBdp rats fed WG diet can be considered as the immunological marker of prediabetic period as demonstrated by Vitali et al.(2000) (Vitali et al., 2000).

No correlation was found between diabetes incidence and serum IFN- γ and TNF- α levels. However, Hussain et al. (1996) found significantly higher serum IFN- γ and TNF- α levels in patients with a recent onset of disease than in the healthy controls (Hussain et al., 1996).

Looking at cytokine and cytokine receptor network, the detectable serum cytokines seems to be excessive cytokines that are free from the cytokine receptors (reviewed by Bienvenu et al., 1998). Also, antibodies to the cytokines are present in the serum which might bind with cytokine, thus act as inhibitors, giving less detectable levels of cytokines (reviewed by Bendtzen et al., 1990). In addition, non-specific cytokine inhibitors, for example plasma proteins affect the

cytokine levels too (reviewed by Bienvenu et al., 1998). There is therefore a possibility of an increase in cytokine receptor or cytokine antibody concentrations leading to lower serum cytokine concentrations in the NTP 2000, WG diet group's rats (for IFN- γ) and WG diet group's rats (for TNF- α). Anti TNF- α antibodies levels usually increase in certain inflammatory diseases (reviewed by Bendtzen et al., 1990).

Although invading lymphocytes producing IFN- γ and TNF- α cytokines are suggested as primary source of these cytokines in type 1 diabetes (reviewed by Cnop et al., 2005), we speculate that the main source of the serum IFN- γ levels is local production of IFN- γ in the pancreas, or small intestine or peripheral blood mononuclear cells whereas a major source of serum TNF- α is peritoneal macrophages and circulating blood monocytes.

We observed that most of the diabetic rats had detectable serum IFN- γ and detectable TNF- α levels. However, Ng et al. (1999) reported only 31.7% and 22 % IDDM patients had IFN- γ and TNF- α cytokine detectable in their serum respectively (Ng et al., 1999).

No correlation between serum IFN- γ and TNF- α levels were found for the WG, WGGSLF and HCGSL diets. However, for rats fed HC and NTP 2000 diets treatments, elevation in TNF- α levels was related to corresponding reduction in IFN- γ levels.

In conclusion, serum IFN- γ and TNF- α assessment suggests GSLEE elevates serum IFN- γ concentration in BBdp rats during IDDM pathogenesis.

Chapter 7. Discussion- Material and Methodological Aspects.

In the present study, we demonstrated the electrophoretic bands having masses corresponding to the gliadin proteins' masses suggesting presence of wheat gliadins in CM soluble sphingolipid enriched extract. We found an insignificant increase in pancreatic lymphoid tissue aggregates; jejunal mucosal thickness and sera IFN- γ concentrations in the HCGSL dietary treatment group's BBdp rats when compared to the HC diet group's rats. However, contrary to our expectation, insulinitis score, jejunal infiltration scores, jejunal CD4+, $\gamma\delta$ TCR+ cell count and serum TNF- α concentrations did not increase in the HCGSL diet group's rats when compared to the HC diet group's rats, owing to the presence of GSLEE and its diabetogenic potential due to gliadin. In contrast, the serum TNF- α concentration was found to be the highest in the HC diet group rats among all the dietary groups. The NTP and WG diets had higher insulinitis scores and higher CD4+ cell counts and lower $\gamma\delta$ TCR+ cell counts in the jejunum of BBdp rats and higher serum IFN- γ levels than what was found due to the HC diet.

Although our findings shed light on the mechanism behind NTP and WG induced diabetes in Shi, 2003 study (M.S. Thesis, Virginia Tech), our results failed to elucidate the mechanism of by which gliadin specific sphingolipid enriched extract induced type 1 diabetes.

There could be quite a few possible explanations for these discrepancies and most of them may relate to material and methodological aspects of our study. First it should be noted that the gliadins present in the CM fraction were not pure gliadin or tryptic digest of gliadins, rather it was present in the

sphingolipid enriched fraction. There are possibilities about different interactions between gliadins and lipids (Huang et al, 2006), might affect the final outcome of the immune system activation potential of the gliadins. Also, the content of the gliadins in the sphingolipid enriched extract was extremely small, since the insoluble residue after first CM extraction was put under second CM extraction while attempting to obtain as much lipid as possible and further Folch wash was employed on the supernatants of two CM extraction to remove water insoluble non-lipid components (Shi, 2003-M.S. Thesis, Virginia Tech). Further a small quantity of sphingolipid enriched extract, 44.4 grams was added to the HC diet, making the gliadin content in the entire HC diet extremely small.

It is probable that wheat glutenin is present in the residue, which is believed to be a diabetogen (reviewed by Scott et al, 1996), might have also contributed to some inconsistent results like significantly higher serum IFN- γ levels in the WGGSLF diet.

In addition, we carried out a retrospective SDS-PAGE analysis of the CM soluble wheat gluten extract and the sphingolipid enriched extract, which were stored for more than 2 years at 4 ° C. Thus, the storage period and storage conditions might have contributed to the reduced content of gliadins in the sphingolipid enriched extract owing to the less intense bands in electrophoresis. Similarly, we carried out a cytokine assay on the frozen sera samples which were stored at -80 ° C for a period. Cytokine sera concentrations may be affected by the length of storage period and storage condition. However, according to published literature, biological samples need to be stored at -80 C during

preanalytical phase (reviewed by Bienvenu et al., 1998). In one published study, the investigators carried out a retrospective assay for cytokine concentration with sera samples stored at -70°C (Burke et al., 1994), in the same manner we conducted.

Similarly our tissue samples were stored at -80°C for more than one year period which might have deteriorated the quality of tissues and that may be one of the reason that even repeated immunohistochemistry assay on few of our tissue specimen resulted into high background staining.

Finally, we didn't had H & E stained slides or the jejunal tissue specimens from all the BBdp rats from five different dietary treatments in order to conduct the jejunal tissue analysis. For instance, we had just 5 tissue specimens from the NTP 2000 group out of 19 BBdp rats. Inadequate and small sample sizes might have affected the overall results by decreasing statistical power.

Most of these type of feeding trials have been conducted from 150 days (reviewed by Scott, 1996)-175 days (Scott et al., 1985) in BB rats to 320 days of age in NOD mice (Funda et al., 1999) and the results seems varying with the time period of the study. The fact that all the required material (except samples of SDS-PAGE) for our study have been obtained between 63- 125 days might have affected the final results of our study in comparison of the results of other similar type of studies.

Irrespective of the retrospective analysis of stored samples and inadequate sample sizes, our results successfully pointed towards gliadins from

the CM soluble sphingolipid enriched extract as one of the diabetogenic constituents.

Chapter 8. Summary and Conclusions, Significance and Suggested Future Investigations.

Summary and Conclusions

The findings of this present study are as follows.

1. SDS-PAGE analysis of the CM soluble extract of wheat gluten and the CM soluble gliadin specific sphingolipid enriched extract (GSLEE) showed electrophoretic patterns with masses corresponding to the masses of gliadin proteins of wheat gluten suggesting the presence of gliadins in the CM soluble sphingolipid enriched extract.
2. Addition of GSLEE to the HC diet did not affect the insulinitis score significantly as compared to the control diet HC. However, insignificant decrease in insulinitis score and lymphoid aggregate content was observed after removing the GSLEE from the wheat gluten diet as compared to the WG diet alone.
3. There were no significant differences in jejunal CD4+ cells, $\gamma\delta$ TCR+ and in jejunal infiltration scores, epithelial erosion, mucosal thickness and jejunal villi height in BBdp rats due to the presence of GSLEE in the HC diet. Removing GSLEE from the WG diet did not decreased CD4+ and $\gamma\delta$ TCR+ cell count significantly when compared to the WG diet groups' rats.
4. Although significant increase in the sera IFN- γ concentrations in HCGSL diet have been found as compared to in the control HC diet, removing GSLEE from the WG diet accounted for a significant increase in serum IFN- γ .
5. Contrary to our hypothesis, the HCGSL diet insignificantly suppressed sera

TNF- α levels when compared to the HC diet. Similarly omitting GSLEE from the WG diet insignificantly elevated sera TNF- α concentrations in BBdp rats.

6. The insulinitis score, jejunal CD4+ cell counts, $\gamma\delta$ TCR+ cell counts, jejunal infiltration scores are higher in BBdp rats from NTP 2000 and WG diets than in BBdp rats from other dietary treatment groups, which explain higher diabetes incidence as a results of these diets in the Shi, 2003 study (M.S. Thesis). Further, these results suggest that the NTP 2000 and WG diets may induce type 1 diabetes by altering pancreatic and jejunal histopathology.
7. The presence of lipid fractions in GSLEE must have acted as immunosuppressants in type 1 diabetes resulting in insignificant differences in jejunal entropathy parameters (mucosal thickness, epithelial erosion and jejunal villi height), jejunal CD4+, jejunal $\gamma\delta$ TCR+, pancreatic lymphoid aggregate content and sera TNF- α cytokine concentrations. Also, there is a possibility that gliadins might have formed a complex with lipids giving a new protein-lipid complex, which is capable of activating the immune system or changing the IFN- γ and TNF- α serum cytokine concentrations through a totally different mechanism.
8. Finally, the findings of this study reemphasized the presence of some diabetogenic constituent in the residue after CM extraction which changes the jejunal histopathology and sera IFN- γ and TNF- α cytokine concentrations, and thus may induce type 1 diabetes.

Significance of these Studies

The present study aimed to investigate the mechanism of pathogenesis of type 1 diabetes by the CM soluble gliadin constituent from GSLEE, in particular change in pancreatic and jejunal histopathology and change in serum IFN- γ and TNF- α cytokine concentrations. Our results suggest that wheat gliadin proteins are present in the CM soluble sphingolipid enriched extract of wheat gluten. Jejunal enteropathy parameters (except infiltration) and serum TNF- α cytokine levels were found to be unaffected by the GSLEE, implying that the immunosuppressant activity of sphingolipids counteracted the immunoactivation potential of gliadins in the CM soluble sphingolipid enriched extract. It also pointed toward the possibility of formation of a protein-lipid complex that may exert effects through a totally different mechanism.

The diabetogenic potential of CM soluble gliadin enriched fraction could be confirmed in *in vivo* studies. Once the CM soluble gliadins are confirmed as dietary diabetogen, these proteins could be avoided from the diet of the people who are at high risk for IDDM.

It may be possible just to remove gliadins from CM soluble sphingolipid enriched extract to obtain an exclusive sphingolipid extract. The therapeutic potential of this exclusive sphingolipid enriched extract could be investigated. Furthermore, this exclusive sphingolipid extract could be used as a part of the diet of the individuals at high risk for type 1 diabetes as a preventive measure. The gut immune system could be targeted with this exclusive sphingolipid

extract, which will act as a gut immune system suppressant and as an intervention strategy.

The residue remaining after CM extraction could be analyzed for the presence of possible dietary diabetogens and could then be removed from the diet of individuals who are at high risk for type 1 diabetes.

Of great importance, elevation in serum IFN- γ could be used as an indicator of the prediabetic period when an individual is put on a specific diet.

In conclusion, the improved knowledge of type 1 diabetes pathogenesis due to CM soluble GSLEE allows us to envision several preventive and therapeutic alternatives.

Suggested Future Investigations

Although our study suggested that the gliadin specific sphingolipid enriched extract (GSLEE) possesses diabetogenic activity, many questions arise. It is our expectation that this study provide foundation for other future studies.

1) Are α and γ gliadins present in the CM soluble extract of wheat gluten?

Our SDS-PAGE analysis suggested the presence of gliadin proteins in the CM soluble wheat gluten extract and the sphingolipid enriched extract. However, due to the complex structure of gliadin proteins and their heterogeneity and since all subtypes are structurally and chemically similar, it was difficult to determine the individual gliadin subtypes by one dimensional SDS PAGE. Two dimensional methods like two-dimensional gel electrophoresis with tandem mass spectrometry method described by Mamone et al. (2005) (Mamone et al., 2005) or RP-HPLC and SDS-PAGE along with protein reference map developed by N-terminal sequencing or mass spectrometry procedure by DuPont et al. (2005) (DuPont et al., 2005) could be particularly useful for α gliadin and γ gliadin detection. Western blotting could be useful, too. Also, method of immunoblotting with the sera as described by Rocher et al. (1995) (Rocher et al., 1995) obtained from IDDM patients may provide clear evidence about the CM soluble gliadins specific peptide as immunogens in type 1 diabetes.

2) Which cell type contributed to CM soluble gliadin induced serum IFN- γ and TNF- α cytokine concentration? –in vitro assay

Future studies could determine IFN- γ and TNF- α production in cell culture of PBMCs, peritoneal macrophages, and infiltrated lymphocytes from small

intestinal and pancreatic islets i.e.- CD4+ T cells, CD8+ T cells or /and respective cell lines after incubation and activation of these cells or cell lines with CM soluble gliadins by following the experimental procedure of Jelinkova et al. (2004) and van de Wal et al. (1999) (Jelinkova et al., 2004 and van de Wal et al., 1999). The results will elucidate the cell type responsible for respective serum cytokine production. Currently no data exist suggesting dietary diabetogens or cell types responsible for the cytokine production due to those dietary diabetogens.

3) *What constituent in residue after CM extraction is diabetogenic?*

Wheat gluten is a heterogenous mixture of gliadins and glutenins. SDS-PAGE, two-dimensional gel electrophoresis with mass spectrometry, a procedure by Mamone et al. (2005) (Mamone et al., 2005) or RP-HPLC and SDS-PAGE along with protein reference map/ mass spectroscopy procedure described by DuPont et al. (2005) (DuPont et al., 2005) could be used to test the presence of glutenins in the residue as well. Western blotting could be employed to look at the presence of glutenins in the residue. In a similar way, the immunoblotting with sera from IDDM patient could provide evidence of glutenin peptides as immunogens in type 1 diabetes. This result of this study may be able to define the mechanisms of diabetogenic activity, jejunal histopathology induction potential and IFN- γ synthesis stimulation potential of the residue after CM extraction which showed diabetogenic activity, increased serum IFN- γ levels and altered jejunal histopathology.

4) Does CM soluble gliadin enriched fraction from the CM soluble extract of wheat gluten act as diabetes trigger?.

First, Coleman et al. (1990) and Shi, 2003 (M.S. Thesis) have shown the diabetogenic potential of CM soluble extracts of cereal based diet, in the same direction our findings suggests that CM soluble gliadins possess diabetogenic activity. Further studies need to separate CM soluble gliadins fraction from the CM soluble extract of wheat gluten, which is obviously lipid enriched. Also, after obtaining the CM soluble gliadin enriched extract, its diabetes trigger activity could be assessed in *in vivo* study. The result of this investigation will extensively characterize the CM soluble gliadin enriched fraction as a diabetogen.

5) *In vitro* mucosal immune responses to CM soluble gliadins in type 1 diabetes.

In vitro cellular mucosal immune responses to CM soluble gliadins could be evaluated by culturing biopsy fragment of jejunum of IDDM patients with CM soluble gliadins by the experimental protocol described by Auricchio et al. (2004) (Auricchio et al., 2004). To determine the humoral responses to CM soluble gliadins, intestinal mucosal humoral immune cells like IgG+ cells, IgM + cells in the small intestine could be assessed by using experimental protocol described by Funda et al. (1999) (Funda et al., 1999). This study would successfully focus on the immune mechanism of CM soluble gliadin induced type 1 diabetes.

6) Do CM soluble gliadins exert proinflammatory or anti-inflammatory cytokine biases in the small intestine and pancreas?

RT-PCR could be used to quantify mRNA expression levels of IFN- γ , TNF- α or IL-4, IL-10 in the small intestine, the peyer's patches and pancreatic islet by using the experimental method of Flohe et al. (2003) (Flohe et al., 2003) after feeding CM soluble gliadins to the BBdp rats vs a diabetes protecting diet. This will help to make it clear whether CM soluble gliadins are able to modify the cytokine expression in type 1 diabetes.

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Appendix A

H & E Stained Pancreatic Tissue Slides (obtained from Shi, 2003 study-M.S. Thesis, Virginia Tech).

Table 1. Total number of H&E stained pancreatic tissue slides from 5 different dietary treatment groups.

Abbreviations for the dietary feeding group	Diet	Number of BBdp rats with H&E stained pancreatic tissue specimen slides
A	HC	20
B	NTP 2000	19
C	WG	18
D	WGGSLF	17
E	HCGSL	17
		Grand total = 91

1. HC-Hydrolyzed casein based diet, the negative control.
2. NTP 2000-National toxicology program 2000 diet, the positive control.
3. WG- Wheat gluten based diet.
4. WGGSLF- Wheat gluten based with the gliadin specific sphingolipid enriched extract removed from the diet.
5. HCGSL- Hydrolyzed casein based diet with the gliadin specific sphingolipid enriched extract added to it.
6. A, B, C, D, E – Abbreviations used for the respective dietary treatments.

Appendix B

***H & E Stained jejunal tissue Slides and Frozen Jejunal Tissue Specimens
(obtained from Shi, 2003 study-M.S. Thesis, Virginia Tech).***

Table 2. Total number of H&E stained jejunal tissue slides and frozen jejunal tissue specimens of BBdp rats from 5 different dietary treatment groups.

Abbreviations for the dietary feeding group	Diet	Number of Bbdp rats with H&E stained jejunal specimen slides	Number of BBdp rats with jejunal tissue specimen
A	HC	16	15
B	NTP 2000	5	5
C	WG	9	8
D	WGGSLF	9	7
E	HCGSL	13	11
		Grand total = 52	Grand total = 46

1. HC-Hydrolyzed casein based diet, the negative control.
2. NTP 2000-National toxicology program 2000 diet, the positive control.
3. WG- Wheat gluten based diet.
4. WGGSLF- Wheat gluten based with the gliadin specific sphingolipid enriched extract removed from the diet.
5. HCGSL- Hydrolyzed casein based diet with the gliadin specific sphingolipid enriched extract added to it.
6. A, B, C, D, E – Abbreviations used for the respective dietary treatments.

Appendix C

3. BBdp Rat's Sera Samples (obtained from Shi, 2003 study-M.S. Thesis, Virginia Tech).

Table 3. Total numbers of sera sample of BBdp rats from 5 different dietary treatment groups.

Abbreviations for the dietary feeding group	Dietary group	Number of BBdp rats with sera samples
A	HC	20
B	NTP 2000	16
C	WG	17
D	WGGSLF	18
E	HCGSL	17
		Grand total = 88

1. HC-Hydrolyzed casein based diet, the negative control.
2. NTP 2000-National toxicology program 2000 diet, the positive control.
3. WG- Wheat gluten based diet.
4. WGGSLF- Wheat gluten based with the gliadin specific sphingolipid enriched extract removed from the diet.
5. HCGSL- Hydrolyzed casein based diet with the gliadin specific sphingolipid enriched extract added to it.
6. A, B, C, D, E – Abbreviations used for the respective dietary treatments.

Appendix D

The BCA™ Protein Assay (for quantification of proteins in the CM soluble extract of wheat gluten and the CM soluble sphingolipid enriched extract).

1. Label centrifuge tube with name, date, experiment number and name of the sample.
2. Add 5 grams of chloroform methanol wheat gluten extract or sphingolipid enriched extract into the tube.
3. Evaporate the organic solvent from the extract using liquid nitrogen for 30 minutes.
4. Add 5 ml of extraction buffer to the above extract.
5. Homogenize the resulting mixture with homogenizer (VIRTIS-VIRTISHEAR, The Virtis Company Inc. NY).
4. Incubate the homogenized mixture for 2 hours at 65 °C in water bath.
5. After incubation, centrifuge the mixture for 5 minutes and use the resulting supernatant for the protein assay using BSA as a standard.
6. Prepare BSA standards by diluting BSA stock to 1000 µg/ml.
7. Prepare standards and sample as below.

Table 1. BSA standard and sample (chloroform methanol soluble wheat gluten extract).

Tube	Volume of BSA standard (µl)	Volume of sample (µl)	Volume of H₂O (µl)	Presume protein content (µg/ml)
Standard 1	0		50	0
Standard 2	5		45	100
Standard 3	10		40	200
Standard 4	15		35	300
Standard 5	20		30	400
Standard 6	25		25	500
Sample A1		10	40	200
Sample A2		20	30	400

8. Use sample to working reagent ratio as 1:20.
9. Prepare BSA working reagent by mixing 50 parts of BCA Reagent A and 1 part of BCA reagent B.
10. Add 1ml of this BSA working reagent to each standard and sample and mix well.
11. Incubate all the tubes in water bath at temperature 37 °C for 30 minutes.
12. Read the resulting mixture using a spectrophotometer (BECKMAN COULTER DU 530) at 562 nm.
13. Determine protein concentration based on standard curve.

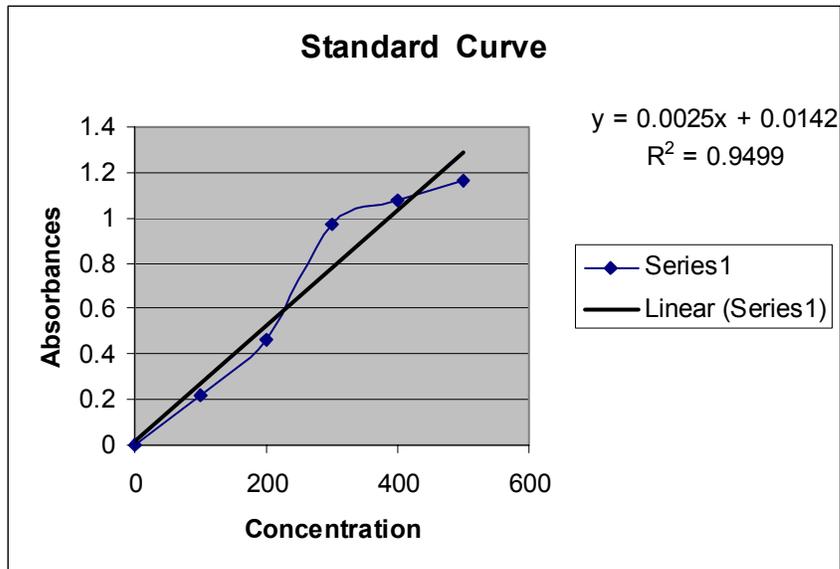
Estimating the protein concentration in CM soluble extract of wheat gluten sample.

Table 2. Protein assay-absorbances (CM soluble wheat gluten extract).

Tube	Absorbances
Standard 2	0.219
Standard 3	0.467
Standard 4	0.969
Standard 5	1.075
Standard 6	1.163
Sample A₁	0.237
Sample A₂	0.528

Table 2. BSA Std Concentrations vs Absorbances (CM soluble wheat gluten extract).

BSA Std Concentration	Absorbance's
0	0
100	0.219
200	0.467
300	0.969
400	1.075
500	1.163



1) Protein concentration (sample A₁) –

$$y = 0.0025x + 0.0142$$

$$0.237 = 0.0025x + 0.0142$$

$$x = 89.12$$

Dilution for sample A₁ is 5 times

$$\text{Protein concentration (sample A}_1\text{)} = 89.12 * 5 = 445.6 \mu\text{g/ml}$$

2) Protein concentration (sample A₂) –

$$y = 0.0025x + 0.0142$$

$$0.528 = 0.0025x + 0.0142$$

$$x = 205.52$$

Dilution for sample A₂ is 5/2 times

$$\text{Protein concentration (sample A}_2\text{)} = 205.52 * 5/2 = 513.8 \mu\text{g/ml}$$

Concentration of proteins in CM soluble wheat gluten extract (Average of sample A₁ and A₂) = $445.6 + 513.8 = 959.4/2 = 479.7 \mu\text{g/ml}$

Appendix E

SDS-PAGE Assay (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis for detection of gliadin proteins in the CM soluble extract of wheat gluten and CM soluble sphingolipid enriched extract).

1. Determine the protein concentration in the CM soluble extract of wheat gluten and the CM soluble sphingolipid enriched extract. 10 µg -25 µg of protein is more than enough for the SDS-PAGE.
2. Label the microcentrifuge tube with name, date, experiment number and name of the sample.
3. Prepare running buffer (1X Tris/ Glucine buffer) by mixing 900 ml of deionized water and 100 ml of 10 X stock buffer. Store it at 4 °C until used for assay.
4. Use Mini-Protein III (BIO-RAD) apparatus with mini-acrylamide gels - ready gel 18% Tris-HCl, 10 well and 30 µl comb (precast gel for polyacrylamide electrophoresis) for the separation of proteins.
5. Take 18% ready gel out of 4 °C, rinse with distilled water. Wash wells with distilled water, remove the extra distilled water from the well by syringe. Then again wash the wells with the running buffer in the same manner.
6. Assemble the gel sandwich box and rinse it with the running buffer. Make sure there is no leaking by pouring the approximately 125 ml of running buffer into the upper chamber
7. Put the sandwich box in the lower chamber and pour approximately 400 ml of running buffer into it
8. First prepare the CM soluble wheat gluten extract sample and the CM soluble

sphingolipid enriched extract sample in the same way as prepared for protein assay by employing first evaporation of organic solvent from the extract, then extraction with extraction buffer, then homogenization, incubation in water bath and finally centrifugation to obtain the supernatant.

9. Prepare protein samples for SDS-PAGE assay by adding 50 μ l of the non-reducing sample buffer to 50 μ l of CM soluble wheat gluten and sphingolipid enriched extract samples separately in separate micro centrifuge tubes.
10. Load approximately 60-70 μ l of this resulting mixture into the ready gel in separate well along with 10 μ l of standard (precision plus proteinTM standard).
11. Perform electrophoresis along with protein markers at 180 V, 100 mA for 50 minutes at room temperature.
12. Wash the gel with ultrapure water for 5 minutes and immediately stain with 20 ml GelCode Blue Stain Reagent solution for 1 hour.
13. Destain the gel with ultrapure water.
14. Employ several water changes for a 1-2 hour period for optimal results, i.e.-this step enhances the stain sensitivity as weak protein bands continue to develop.
15. Scan the gel to obtain the bands by using the scanner Alpha Image 2000 connected to computer, equipped with Alpha Imager 2000 3.3 b software (Alpha Innotech Corporations).
16. Determine the molecular weights of the resulting protein bands in kD by comparing them with the molecular weight of marker proteins.
17. Observe the resulting band intensities to assess the protein content.

Appendix F

Immunohistochemistry Assay (for CD4 and $\gamma\delta$ TCR in jejunal tissue specimens).

1. First mark poly-L-lysine coated slides with tissue name, tissue number and date.
2. Run healthy, fresh rat spleen tissue specimens and rat small intestine tissue specimens as a positive control for CD4 immunohistochemistry and a healthy, fresh rat small intestine tissue specimen as a positive control for $\gamma\delta$ TCR immunohistochemistry.
3. Prepare the tissue specimens by OTC method i.e.- first add tissue freezing media into the OTC cup and then put the tissue specimen in the middle of the OTC cup, add more tissue freezing media to it so that the entire tissue gets covered by it and then keep OTC cup on the dry ice for 15-20 minutes.
4. Place the OTC block containing tissue specimens on a cryostat specimen disk and then adjust the OTC block position in such a way that it is aligned with the blade of the instrument.
5. Cut tissue sections of the jejunal tissue specimens by using a cryostat (MicroM – HM 505 N) at 8 μ m thickness and mount on the marked poly-L-lysine slides with two sections of each tissue on one slide.
6. Once the sections are dried for 5 minutes, fix them with 100% histology grade acetone in IHC jar (approximately 250 ml).
7. Allow these tissue sections to dry for 5 minutes.
8. Transfer these slides with tissue sections on it to the slide box and store them

- at -20 °C in a zip lock bag until used for immunohistochemistry.
9. Perform the immunohistochemistry assay at room temperature.
 10. Prepare fresh 1X PBS by mixing 100 ml 10X PBS with 900 ml nanopure water.
 11. For immunohistochemistry, first equilibrate the tissue slides to room temperature for 1 minute.
 12. Incubate tissue slides in 1X PBS buffer in IHC jar (approximately 250 ml) twice for 5 minutes each.
 13. Transfer the tissue slides in the humidity chamber and immediately incubate them with the blocking buffer (1X PBS with 10% normal goat serum) @ 0.7ml of blocking buffer/slide for 30 minutes.
 14. After eliminating the blocking buffer, incubate the tissues at room temperature for 4 hours with primary antibody diluted in 1X PBS with 5% normal goat serum @ of 0.7ml of diluted primary antibody/slides. The slides should be in the humidity chamber to avoid drying for these 4 hours.
 15. Use concentration of 3 µg/ml for CD4 primary antibody, whereas use primary antibody concentration of 4 µg/ml for γδTCR. (These are the optimum primary antibody concentrations for our jejunal tissue specimens, these optimum concentrations are obtained from preliminary immunohistochemistry assay conducted with the jejunal tissue specimen using different primary antibody concentrations for the respective CD4 and γδTCR molecules).
 16. After incubation, wash the tissue slides twice with 1X PBS in IHC jar (approximately 250 ml).

17. To eliminate endogenous peroxidase activity, transfer the tissue slides to the IHC jar containing 200 ml of methanol with 30% hydrogen peroxide (2.5 ml for CD4 and 2 ml for $\gamma\delta$ TCR) and incubate for 30 minutes.
18. After 30 minutes, wash the tissue slides twice with 1X PBS and place them into humidity chamber to avoid tissue drying.
19. After eliminating excess 1X PBS, incubate the tissue slides with secondary antibody diluted into 1X PBS with 5% normal goat serum @ 0.7ml of diluted secondary antibody/slide and place the slides in them humidity chamber for 1 hour.
20. Use 1/100 dilution and 1/45th dilution of the secondary antibody for CD4 and $\gamma\delta$ TCR respectively (These are the optimum secondary antibody concentrations for our jejunal tissue specimens, these optimum concentrations are obtained from preliminary immunohistochemistry assay conducted with the jejunal tissue specimen using different concentrations of secondary antibody for the respective CD4 and $\gamma\delta$ TCR molecules).
21. After two washes with 1X PBS, incubate the tissues with STRAPTAVIDIN-HRP for 30 minutes.
22. Wash the tissue slides with 1X PBS twice and add DAB-diaminobenzidene substrate on the tissue sections and incubate until color develops (approximately 2 to 10 minutes).
23. Rinse the tissue slides with nanopure water and counterstain with hematoxylin for 1 minute.
24. Again rinse the tissue slides with nanopure water followed by tap water.

25. Finally add immu-mount on the tissue section and covered it with a cover slip.
26. Allow immunostained slides to dry at room temperature and then store at room temperature until used for microscopic analysis.

Appendix G

ELISA Assay (for quantification of IFN- γ and TNF- α concentrations in the rat sera).

1. Conduct ELISA as per manufacturer's recommended protocol – eBioscience, San Diego, CA, California, USA.
2. First design plate template on paper considering standard and number of samples to be analyzed, each in triplet.
3. Coat 96 well NUNC Maxisorp flat-bottom plate with either 100 μ l of anti IFN- γ or 100 μ l of anti TNF- α antibodies in 1X coating buffer (0.1M NaHCO₃).
4. Seal the plate and incubate overnight (12 hrs) at 4 °C.
5. On the next day first prepare fresh washing buffer by adding 0.5 ml of Tween-20 to 1 liter of 1X PBS.
6. Aspirate and wash the wells with 300 μ l of washing buffer followed by inverting and blotting against the absorbent paper to remove any residue.
7. Perform three consecutive washes (4 minutes/wash) with washing buffer.
8. Incubate the plate with 1X assay diluent for 1 hour at room temperature after adding 200 μ l of assay diluent /well and sealing the plate.
9. Again aspirate and wash the plate twice with ~300 μ l of washing buffer.
10. Add 100 μ l of standard/well to appropriate wells followed by 100 μ l of the sample/well as per the plate template design. To obtain the standard curve, perform two-fold serial dilutions of the top standard. Add standards and the samples in triplicate.
11. Seal the plate and Incubate for 2 hours and then aspirate and wash the wells

- 5 times with washing buffer.
12. Add secondary antibody conjugated with biotin, 100 µl/ well, seal the plate and incubate the plate for 30 minutes at room temperature.
 13. Aspirate and wash the wells with washing buffer 5 times.
 14. Add 100 µl of avidin-HRP diluted in 1X assay diluent /well and then seal the plate and incubate for 30 minutes at room temperature.
 15. Repeat 7 washes of washing buffer and then add 100 µl of substrate/well.
 16. Seal the plate and cover the plate with aluminum foil to protect DAB chromogen from light.
 17. Incubate the plate for 15 minutes or until the color develops.
 18. Add 50 µl stop solution (2N H₂SO₄) to each well.
 19. Read the plate with microplate reader µQuant (BIO-TEK Instruments Inc.) at 450 nm wavelength with 570 nm as a reference wavelength.
 20. Obtain the absorbances, standard curve and the corresponding cytokine concentrations in pg/ml with software KC Junior.

Appendix H

Pancreatic Tissue Histopathology Assessment Score Ranking.

1) Lymphocytic infiltration (H&E stained pancreatic tissue slides).

Table 1. % infiltration in pancreatic islet and the corresponding score ranking (magnification 400X).

Score	% Mononuclear cell infiltration
1	1%-20%
2	20%-40%
3	40%-60%
4	60%-80%
5	80%-100%

2) Lymphoid aggregates (H&E stained pancreatic tissue slides).

Table 2. Lymphoid tissue content in pancreatic tissue and the corresponding score ranking (magnification 200X).

Score	Lymphoid aggregate content
1	Normal
2	Medium
3	High

3) Adipose tissues (H&E stained pancreatic tissue slides).

Table 3. Adipose tissue content in pancreatic tissue and the corresponding score ranking (magnification 200X).

Score	Adipose tissue content
1	Normal
2	Medium
3	High

Appendix I

Jejunal Tissue Histopathology Assessment Score Ranking.

1) Lymphocytic infiltration in the epithelium, intraepithelium and payer's patches (H&E stained jejunal tissue slides).

Table 1. % infiltration in jejunal tissue and the corresponding score ranking (magnification 400X).

Score	% infiltration
1	1-33%
2	33%-66%
3	66%-100%

2) Mucosal thickness (H&E stained jejunal tissue slides).

Table 2. Mucosal thickness and corresponding score ranking (magnification 200X).

Score	Mucosal thickness
1	Normal
2	Medium thick
3	Highly thick

3) Epithelial erosion (H&E stained jejunal tissue slides).

Table 3. Epithelial erosion and corresponding score ranking (magnification 200X).

Score	Epithelial erosion
1	Normal epithelium
2	Medium erosion
3	High erosion

4) Jejunal villi height (H&E stained jejunal tissue slides).

Table 4. Jejunal villi height and corresponding score ranking (magnification 200X).

Score	Jejunal villi height
1	Normal-finger like projection
2	Medium
3	Completely flatten

Appendix J

ANOVA Tables (GLM PROCEDURES)

Table 1. Analysis of variance for the insulinitis according to dietary treatments (H& E stained pancreatic tissue slides).

Dependent Variable: IS (Insulinitis score)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	17.10241345	4.27560336	6.81	0.0001
Error	86	54.02008545	0.62814053		
Corrected Total	90	71.12249890			

R-Square	C.V.	Root MSE	IS Mean
0.240464	28.07518	0.79255317	2.82296703

Source	DF	Type I SS	Mean Square	F Value	Pr > F
DIET	4	17.10241345	4.27560336	6.81	0.0001

Source	DF	Type III SS	Mean Square	F Value	Pr > F
DIET	4	17.10241345	4.27560336	6.81	0.0001

Table 2. Analysis of variance for the lymphoid aggregate content according to dietary treatments (H& E stained pancreatic tissue slides).

Dependent Variable: LA (Lymphoid aggregate)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	7.38097374	1.84524343	1.00	0.4124
Error	86	158.75089439	1.84594063		
Corrected Total	90	166.13186813			

R-Square	C.V.	Root MSE	LA Mean
0.044428	106.5841	1.35865398	1.27472527

Source	DF	Type I SS	Mean Square	F Value	Pr > F
DIET	4	7.38097374	1.84524343	1.00	0.4124

Source	DF	Type III SS	Mean Square	F Value	Pr > F
DIET	4	7.38097374	1.84524343	1.00	0.4124

Table 3. Analysis of variance for the adipose tissue content according to dietary treatments (H& E stained pancreatic tissue slides).

Dependent Variable: AT (Adipose tissue)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	6.69683598	1.67420900	2.01	0.1007
Error	86	71.74272446	0.83421773		
Corrected Total	90	78.43956044			

R-Square	C.V.	Root MSE	AT Mean
0.085376	50.68008	0.91335520	1.80219780

Source	DF	Type I SS	Mean Square	F Value	Pr > F
DIET	4	6.69683598	1.67420900	2.01	0.1007

Source	DF	Type III SS	Mean Square	F Value	Pr > F
DIET	4	6.69683598	1.67420900	2.01	0.1007

Table 4. Analysis of variance for the infiltration according to dietary treatments (H& E stained jejunal tissue slides).

Dependent Variable: IF (Infiltration)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	1.50241453	0.37560363	4.37	0.0044
Error	47	4.03835470	0.08592244		
Corrected Total	51	5.54076923			

R-Square	C.V.	Root MSE	IF Mean
0.271156	20.65382	0.29312530	1.41923077

Source	DF	Type I SS	Mean Square	F Value	Pr > F
DIET	4	1.50241453	0.37560363	4.37	0.0044

Source	DF	Type III SS	Mean Square	F Value	Pr > F
DIET	4	1.50241453	0.37560363	4.37	0.0044

Table 5. Analysis of variance for change in mucosal thickness according to dietary treatments (H& E stained jejunal tissue slides).

Dependent Variable: MT (Mucosal thickness)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	1.03557692	0.25889423	1.90	0.1263
Error	47	6.40673077	0.13631342		
Corrected Total	51	7.44230769			

R-Square	C.V.	Root MSE	MT Mean
0.139147	31.47334	0.36920647	1.17307692

Source	DF	Type I SS	Mean Square	F Value	Pr > F
DIET	4	1.03557692	0.25889423	1.90	0.1263

Source	DF	Type III SS	Mean Square	F Value	Pr > F
DIET	4	1.03557692	0.25889423	1.90	0.1263

Table 6. Analysis of variance for epithelial erosion according to dietary treatments (H& E stained jejunal tissue slides).

Dependent Variable: EE (Epithelial erosion)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	2.13675214	0.53418803	1.50	0.2187
Error	47	16.78632479	0.35715585		
Corrected Total	51	18.92307692			

R-Square	C.V.	Root MSE	EE Mean
0.112918	38.84564	0.59762517	1.53846154

Source	DF	Type I SS	Mean Square	F Value	Pr > F
DIET	4	2.13675214	0.53418803	1.50	0.2187

Source	DF	Type III SS	Mean Square	F Value	Pr > F
DIET	4	2.13675214	0.53418803	1.50	0.2187

Table 7. Analysis of variance for jejunal villi height according to dietary treatments (H& E stained jejunal tissue slides).

Dependent Variable: JV (Jejunal villi)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	1.37478632	0.34369658	0.82	0.5190
Error	47	19.70213675	0.41919440		
Corrected Total	51	21.07692308			

R-Square	C.V.	Root MSE	JV Mean
0.065227	38.25854	0.64745224	1.69230769

Source	DF	Type I SS	Mean Square	F Value	Pr > F
DIET	4	1.37478632	0.34369658	0.82	0.5190

Source	DF	Type III SS	Mean Square	F Value	Pr > F
DIET	4	1.37478632	0.34369658	0.82	0.5190

Table 8. Analysis of variance for CD4+ cell count according to dietary treatments (jejunal tissue specimen).

Dependent Variable: CD4+

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	7362.58854	1840.64713	2.33	0.0740
Error	37	29231.84289	790.04981		
Corrected Total	41	36594.43143			

R-Square	C.V.	Root MSE	CD4+ Mean
0.201194	28.56901	28.10782	98.38571

Source	DF	Type I SS	Mean Square	F Value	Pr > F
DIET	4	7362.588537	1840.647134	2.33	0.0740

Source	DF	Type III SS	Mean Square	F Value	Pr > F
DIET	4	7362.588537	1840.647134	2.33	0.0740

Table 9. Analysis of variance for GD ($\gamma\delta$) TCR+ cell count according to dietary treatments (jejunal tissue specimen).

Dependent Variable: GDTCR +

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	717.602883	179.400721	2.54	0.0552
Error	39	2757.112571	70.695194		
Corrected Total	43	3474.715455			

R-Square	C.V.	Root MSE	GD Mean
0.206521	17.50515	8.408043	48.03182

Source	DF	Type I SS	Mean Square	F Value	Pr > F
DIET	4	717.6028831	179.4007208	2.54	0.0552

Source	DF	Type III SS	Mean Square	F Value	Pr > F
DIET	4	717.6028831	179.4007208	2.54	0.0552

Table 10. Analysis of variance for IFN- γ levels (pg/ml) in sera samples of BBdp rats according to dietary treatments.

Dependent Variable: IFN-g (IFN- γ)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	88777.8619	22194.4655	28.73	<.0001
Error	81	62566.0871	772.4208		
Corrected Total	85	151343.9490			
R-Square		C.V.	Root MSE	IFN-g Mean	
0.586597		79.11475	27.79246	35.12930	
Source	DF	Type I SS	Mean Square	F Value	Pr > F
DIET	4	88777.86187	22194.46547	28.73	<.0001
Source	DF	Type III SS	Mean Square	F Value	Pr > F
DIET	4	88777.86187	22194.46547	28.73	<.0001

Table 11. Analysis of variance for TNF- α levels (pg/ml) in sera samples of BBdp rats according to dietary treatments.

Dependent Variable: TNF-a (TNF- α)

Source	DF	Sum of Squares	Mean Square	F Value	Pr > F
Model	4	12.3888266	3.0972067	1.54	0.1980
Error	79	158.6023703	2.0076249		
Corrected Total	83	170.9911970			

R-Square	C.V.	Root MSE	TNF-a Mean
0.072453	76.61125	1.416907	1.849476

Source	DF	Type I SS	Mean Square	F Value	Pr > F
DIET	4	12.38882664	3.09720666	1.54	0.1980

Source	DF	Type III SS	Mean Square	F Value	Pr > F
DIET	4	12.38882664	3.09720666	1.54	0.1980

Vita

Kalpana Thakare, daughter of Mr. Nagoraoji and Mrs. Lakshmi, was born in very small village in India. She has earned her professional degree in Ayurvedic Medicine in 2002 from India. In August 2003, she has started her graduate studies at Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA, working on type 1 diabetes under Dr. Barbeau. Currently she is a predoctoral fellow pursuing her Ph D in Pharmaceutical Sciences. She is planning to work on novel drug delivery and pharmacokinetics for her doctoral dissertation.