Examination of the Effects of a Sphingolipid-Enriched Lipid Fraction from Wheat Gluten on the Incidence of Diabetes in BBdp Rats

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(ABSTRACT)

This study was designed to examine if a sphingolipid-enriched lipid fraction from wheat gluten could affect the incidence of type I diabetes in BioBreeding diabetes prone (BBdp) rats. Wheat gluten was extracted with a chloroform-methanol (CM) mixture to isolate most of the lipids. Isolated lipids were subjected to silica gel column chromatography and saponification to remove most of neutral lipids and phospholipids, leaving behind a lipid fraction enriched in sphingolipids. This sphingolipid-enriched lipid fraction was used in a BBdp rat feeding study. BBdp rats were fed with one of five diets from weaning at 23 days of age until 125 days of age: a hydrolyzed casein based diet (HC), a NTP-2000 standard rodent diet (NTP-2000), a wheat gluten based diet (WG), a sphingolipid-free wheat gluten based diet (WGSLF), and a hydrolyzed casein plus sphingolipid-enriched lipid fraction diet (HC+SL).

The yield of sphingolipid-enriched lipid fraction was about 0.62% of wheat gluten. The content of glycosylceramide in sphingolipid-enriched lipid fraction was increased more than five fold compared to that in total isolated lipids. Rats fed the NTP-2000 diet had the highest incidence of diabetes; while rats on the HC diet had the lowest diabetes incidence. There was no significant difference with regard to the onset age of diabetes among rats in the five diet groups. The addition of sphingolipid-enriched fraction to the HC diet caused a significant increase in the incidence of diabetes in BBdp rats in the first 80 days of the study. However, the ultimate diabetes incidence at day 125 was not changed. The removal of lipids from wheat gluten did not change the diabetes incidence in BBdp rats at any stages of the feeding study. These findings suggest that the sphingolipid-enriched fraction from wheat gluten acted as a possible promoter but not as a trigger of the development of type I diabetes in BBdp rats. There must be something that remains in wheat gluten after chloroform-methanol extraction that serves as a trigger

for type I diabetes in these rodents. Type I diabetes in this animal model for the human disease seems to be caused by multiple factors, most likely, by the interaction of sphingolipids and some other unknown substances in wheat gluten.

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Table of Contents

List of Tables

List of Figures

List of Appendices

Glossary of Terms

A

ANOVA: Analysis of variance

B

BBdp rats: Biobreeding diabetes prone rats

C

CAM: Cell adhesion molecules

CM mixture: Chloroform-methanol (2:1 v/v)

D

DPP IV: Dipeptidyl peptidase IV

E

ELSD: Evaporative light-scattering detector

ESI: Electrospray ionization

F

FAB: Fast atom bombardment

G

GAD: Glutamic acid decarboxylase

GALT: Gut-associated lymphoid tissue

GlcCer: Glucosylceramide

GLP-1: Glucagon-like peptide-1

H

HC diet: Hydrolyzed casein-based diet

HC+SL: Hydrolyzed casein-based diet plus a sphingolipid-enriched lipid fraction from wheat gluten

HLA: Human leukocyte antigen

HPLC: High pressure liquid chromatography

I

IDDM: Insulin-dependent diabetes mellitus

IFN-γ: Interferon-γ

IL-4: Interleukin-4

IL-5: Interleukin-5

IL-6: Interleukin-6

IL-10: Interleukin-10

IL-13: Interleukin-13

M

MAdCAM: Mucosal addressin cell adhesion molecule

MHC: Major histocompatibility complex

MS: Mass spectrometry

N

NKT cells: Natural killer T cells

NO: Nitric oxide

NOD mice: Non obese diabetic mice

NTP-2000 diet: National toxicity program-2000 diet

P

PCR: Polymerase chain reaction

PEG: Polyethyleneglycol

PKC: Protein kinase C

PP2A: Protein phosphatase 2A

T

TCR: T cell receptor

T_c cells: CD8 cytotoxic T cells

T_h cells: CD4 helper T cells

TLC: Thin layer chromatography

TNF-α: Tumor necrosis factor-α

W

WG diet: Wheat gluten-based diet

WGSLF: Wheat gluten based, sphingolipid-free diet

Z

ZDF rats: Zucker diabetic fatty rats

Chapter 1: Introduction

Type I diabetes, also called insulin-dependent diabetes mellitus (IDDM), is mostly diagnosed in children and young adults. This disease occurs predominantly among people of northern Europe origin, while it has very low frequencies among Black, Japanese and Chinese. The incidence rate varies greatly in the world from 30/100,000 in Finland to 0.7/100,000 in Shanghai, China. Statistics showed that the incidence rate has increased 3% per year in the last three decades (Onkamo et al., 1999).

Type I diabetes is an autoimmune disease characterized by the lymphocytic and monocytic infiltration of pancreatic islets. As a result, the insulin-secreting β-cells are gradually destroyed. Since insulin is necessary for the body to utilize glucose, blood glucose homeostasis is not able to be maintained in type I diabetic patients. Type I diabetic patients often suffer from secondary complications such as cardiovascular disease, kidney disease and strokes, which can even cause premature death (Kraine and Tisch, 1999).

Many studies have been performed to investigate the etiology of type I diabetes, but the cause of this disease is still poorly understood. Genetic predisposition is a prerequisite allowing the autoimmune process to progress. However, recent studies show that environmental factors also play a very important role in triggering this disease (Bodansky et al. 1992). Among environmental factors, viral infections and dietary components are most likely to initiate this disease (Akerblom and Knip, 1998).

Animal feeding studies have been used to study the effect of dietary factors in the development of type I diabetes. Non obese diabetic (NOD) mice and Biobreeding diabetes prone (BBdp) rats are used as rodent models in studies because they can development type I diabetes spontaneously which is believed to be etiopathologically similar to humans (Parfrey et al., 1989; Scott, 1996). Among all the rodent diets, wheat-based diets are most diabetogenic, while semi-purified casein- or hydrolyzed casein-based diets have a protective effect (Elliott et al., 1988; Scott et al., 1985; Beales et al., 2002). Funda et al. (1999) reported that the NOD mice on wheat gluten-free diet had a significantly lower incidence of diabetes compared with those on standard rodent diet which was mostly

cereal-based. Moreover, these animals developed diabetes significantly later. Scott et al. (1996) modified standard rodent diet with wheat gluten as exclusive protein source and found that this modified diet caused a highly significant diabetogenic effect ($p<0.00001$). Coleman et al. (1990) found that a 2:1 (v/v) chloroform-methanol (CM) mixture could extract diabetogenic substances from wheat flour, since the addition of this CM extract to the diet significantly increased diabetes incidence in NOD mice. These studies strongly suggest that there must be some substances in wheat which can cause type I diabetes, and these substances are lipophilic.

Sphingolipids are one of the lipid classes which can be extracted by a chloroform-methanol mixture (Christie, 1996). They are widely distributed in foods, including wheat (Sugawara and Miyazawa, 1999). Sphingolipids are hydrolyzed and metabolized in the small intestine and their metabolites are quickly taken up by cells (Schmelz et al, 1994). Ceramide and some other sphingolipids have been shown to play a very important role in cell regulation. These molecules function as second messengers to regulate cell growth and induce apoptosis, i.e. programmed cell death (Pettus et al., 2002). Ceramide is found to be involved in induction of the β-cell dysfunction in cell culture studies. It causes the inhibition of pancreatic β-cell production and mitogenesis (Sjorholm, 1995). It also has a cytotoxic effect on β-cells and can cause β-cell apoptosis. Both exogenously delivered and endogenously synthesized ceramide were found to induce β-cell DNA fragmentation, which is a marker of cell apoptosis (Shimabukuro et al., 1998).

Sphingolipids may be also involved in the disturbance of gut immune system, which is proposed to play a key role in triggering type I diabetes (Kolb and Pozzilli, 1999). Glycosphingolipids, such as glucosylceramide and galactosylceramide, are presented by intestinal epithelial cells to natural killer T (NKT) cells in gut. The activation of NKT cells causes a rapid production of cytokines and activation of the other immune cells in the gut such as CD4 helper T (T_h) cells, CD8 cytotoxic T (T_c) cells, macrophages, etc. (Kolb and Pozzilli, 1999). These immune cells may be released from gut and reach other organs such as the pancreas. It was proposed that β-cell autoreactive lymphocytes in type I diabetes may belong to the gut-associated lymphocytes and may even originate from the gut mucosa (Vaarala, 1999).

Individuals with a genetic predisposition to type I diabetes were found to have higher permeability of the intestinal epithelium which may facilitate the contact of certain food antigens with the mucosal immune system (Kuitunen et al., 2002). This defect increases the susceptibility to type I diabetes in these people. Wheat is the one of the staple foods for humans. The frequent exposure to wheat sphingolipids during infancy may have a negative effect on the gut immune system in diabetes susceptible individuals, which may gradually induce type I diabetes.

Chapter 2: Objectives and Hypothesis

Significance of Study

A wheat-based diet is found to be diabetogenic in rodents (Scott, 1996). Sphingolipids, especially free ceramides, have been used in cell culture studies and found to cause β-cell apoptosis (Shimabukuro et al., 1998). However, so far no studies have been conducted to examine the effect of sphingolipids on the development of type I diabetes in animal feeding studies. This study was designed to examine if wheat sphingolipids were responsible for the diabetogenic potency of wheat. A sphingolipids-enriched lipid fraction was extracted from wheat gluten and it was used in BBdp rats feeding study to examine its effect on the incidence of type I diabetes in these animals. If sphingolipids in wheat gluten could be identified as food diabetogens, they could be possibly removed by certain bioengineering techniques from the food supply of the diabetes susceptible individuals, then type I diabetes might be preventable.

Specific Objectives

To determine if a sphingolipid-enriched lipid fraction from wheat gluten could increase the incidence of type I diabetes in BBdp rats.

Research Hypotheses

The following null hypothesis was tested in this study:

H_0: There will be no significant difference in the incidence of diabetes in BBdp rats due to the presence or absence of wheat sphingolipid-enriched lipid fraction in the animals' diet.

Basic Assumptions

The followings are basic assumptions made by the investigators:

1. Rats were well fed and hydrated;
2. Rats were disease and pathogen free; and

3. The extraction of sphingolipid-enriched lipid fraction from wheat gluten did not change the chemical and biological properties of sphingolipids.

Chapter 3: Review of Literature

Type I Diabetes

Type I diabetes, also called insulin-dependent diabetes mellitus (IDDM), is one of the most common chronic childhood diseases. It accounts for about 10% of all diabetes, and affects approximately 1.4 million people in the U.S. and 10-20 million globally (Rewers, 1991; Libman et al., 1993). The incidence of type I diabetes varies extraordinary in different areas, with higher incidence in northern Europe and lower incidence in most Asian countries. Finland has the highest rate of type I diabetes in the world, with more than 30/100,000 children aged 0-14 years affected by this disease every year. The incidence of this disease is increasing. In the last three decades, the rate of overall increase is about 3.0% per year, with higher relative increase in the populations with a low incidence. If this trend continues, the incidence will be 50/100,000 in Finland and it will exceed 30/100,000 per year in many other areas by the year 2010 (Onkamo et al., 1999).

Type I diabetes is an autoimmune disease caused by the destruction of insulin-producing β cells located in the pancreatic islets of Langerhans (Atkinson and Maclaren, 1990). This disease is characterized by hyperglycemia, hypoinsulinemia, and mononuclear cell infiltration of the islets. Since insulin helps cells take up glucose, this disease can cause glucose accumulation in the blood. The kidney then works overload to filter the glucose into the urine. Consequently, the body breaks down stored fat and protein to provide fuel to the cells. Type I diabetic patients usually suffer from secondary complications, such as cardiovascular disease, kidney disease, neuropathy, strokes, etc, which can cause coma and even premature death. Therefore, patients with type I diabetes have to depend on insulin administration to control blood glucose (Kraine and Tisch, 1999).

The autoimmune attack in type I diabetes involves both cellular and humoral changes (Beyan et al., 2003). However, it is mainly T-cell mediated which requires both CD4 helper T (T_h) cells and CD8 cytotoxic T (T_c) cells (Tisch and McDevitt, 1996). CD4 T_{h1} cells are found to be the effector cells in this disease. They mainly secrete pro-inflammatory cytokines such as interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α),

which are destructive to β cells. These effector T_{h1} cells are regulated by CD4 T_{h2} cells, which mainly secrete protective cytokines such as interleukin-4 (IL-4), IL-5, IL-6, IL-10 and IL-13 (Delovitch and Singh, 1997). During onset and progression of type I diabetes, the cytokine pattern shifts from a Th_2 to Th_1 type (Almawi et al., 1999). Healthy β cells express low levels of major histocompatibility complex (MHC) class I molecules and no class II molecules, while in diabetic β cells, both class I and class II molecules are highly expressed (Elgert, 1996). These MHC molecules present autoantigens in islets to the T cells, thus promoting and exacerbating the autoimmune attack. Some autoantigens in pancreatic islets have been identified, such as glutamic acid decarboxylase (GAD), insulin, gangliosides, etc (Atkinson and Maclaren, 1990). Overproduction of nitric oxide (NO) induced by cytokines is also found in diabetic islet β-cells, and has been shown to be closely related to the inhibition of insulin release and ultimate β-cells destruction (Kaneto et al., 1995).

Rodent Models of Type I Diabetes

Two rodent models are typically used in type I diabetes studies: non-obese diabetic (NOD) mice and biobreeding diabetes prone (BBdp) rats. These animals can develop type I diabetes spontaneously and they have many similarities to humans in the aspects of type I diabetes, such as genetic predisposition, autoimmune process, presence of severe lymphocytic insulitis (i.e. lymphocytic infiltration), metabolic disorder, etc (Leiter et al., 1990; Parfrey et al., 1989; Marliss et al., 1982).

NOD mouse is a well-characterized animal model of type 1 diabetes. The NOD mouse was established by Shionogi Research Laboratories in Japan in 1980 (Makino et al., 1980). At the age of 3-4 weeks, the pancreatic islets of Langerhans in NOD mice are infiltrated first by dendritic cells and macrophages and then by T cells (CD4 and CD8) and B cells. This stage is then followed by a slow but progressive destruction of β-cells by 4-6 months of age. Unlike humans, gender influences disease incidence in NOD mice, with higher disease frequencies in females. By 30 weeks of age, 80-90% of female mice develop overt diabetes while this disease only occurs in 10-40% of males (Delovitch and Singh, 1997). As in humans, diabetes susceptibility in the NOD mice is inherited through

multiple genes, with the determining genes within the class II MHC region (Hattori et al., 1986).

The BBdp rat was developed in 1974 in BioBreeding Laboratories of Canada Ltd. This strain of rat is very susceptible to developing pulmonary or other infections caused by common bacteria and viruses, so it is necessary to keep these rats in a germ-free environment (Marliss et al., 1982). As in the NOD mice and humans, genetic susceptibility for the development of type 1 diabetes in the BBdp rat is also polygenic. At least three genes have been reported to be diabetes associated: a lymphopenia gene Iddm1, a MHC-linked gene Iddm2 and an unmapped gene Iddm3 (Scott, 1996). As in the human, a pronounced pancreatic insulitis is also present in newly diagnosed diabetic BBdp rats. Insulitis begins with macrophages and later T cells (both CD4 and CD8), B cells, NK cells and dendritic cells (Parfrey et al., 1989; Scott, 1996). The incidence of diabetes in males and females are found equally at about 50-60%. BBdp rats develop diabetes around puberty and adolescence between 55 to 140 days with an average onset age at about 90 days (Scott, 1996).

Genetic Susceptibility

Genetic susceptibility to type I diabetes is a prerequisite that allows the autoimmune process to progress. Genetic abnormalities in the MHC class II molecules contribute to the susceptibility of type I diabetes (Atkinson and Maclaren, 1990). In mice, two types of class II MHC proteins are encoded: IE and IA, which are important in normal suppressor activity and the recognition of antigens by T cells respectively. MHC in human is also called human leukocyte antigen (HLA) complex. HLA histocompatibility class II regions have three loci – DP, DQ and DR (Elgert, 1996). The susceptibility determining genes in type I diabetes are located in the DQ region (Reijonen et al., 1990). If the position 57 on DQ B chain is occupied by negatively charged amino acid such as aspartic acid, the possibility of developing diabetes is low, while the non-charged amino acids such as valine or serine in that position can raise the risk of diabetes (Eisenbarth and Lafferty, 1996). Atkinson and Maclaren (1990) proposed a “hotdog” model to demonstrate the importance

of the amino acid in position 57 in DQ B chain. In this model, DQ A and B chain combine together to make a shape of hot dog bun with the inner cleft of which binds the antigen. The 57th amino acid of B chain is on the surface of the cleft at a spot accessible to both antigen and T cell receptor (TCR). The property of amino acid in that position determines the conformation of the cleft. A cleft with neutral amino acid at that position may bind tightly to some autoantigens which are responsible for the type I diabetes. This tight binding may increase the possibility of recognition of autoantigens by T cells.

However, genetic predisposition is not the only reason for the development of this disease. The concordance rate for type I diabetes in identical twins is less than 50% (Barnett et al, 1981), suggesting that environmental factors also play a very important role in the development of type I diabetes. It is believed that the interaction between genetic susceptibility and environment factors contributes to the onset of this disease.

Environmental Factors

There is increasing evidence showing the important role of environmental factors in etiology of type I diabetes. For instance, it is found that there is a steady increase in diabetes incidence worldwide in recent decades. In Finland, the incidence was increased from 13/100,000 in 1953 to 36/100,000 in the early 1990s in the children 0-15 years old. This sharp increase was most likely caused by environmental factors (Akerblom and Knip, 1998). Strong evidence for the environmental effect also comes from studies on migrant populations from a low-incidence area to a high-incidence area. Bondansky et al. (1992) studied a group from south Asia that migrated to the United Kingdom (UK) for a period of twelve years from 1978 to 1990. They found that the offspring of this migrant population had an increasing incidence of type I diabetes which was approaching that of the UK population. The diabetes incidence in children (0-16 years old) in that group was only 3.1/100,000 per year in 1978-1981, while it reached 11.7/100,000 in 1988-1990. However, the incidence remained stable at 10.5/100,000 per year in native children. Feltbower et al (2002) examined type I diabetes in children (0-14 years old) also in migrant population from south Asia to UK but their study last a longer period from 1978 to 1998. The results from this study were in concordance with those from the first research group. A steady

increase in diabetes was also found which was reaching that of the native population. The authors concluded that genetic factors could not explain such a rapid change in diabetes incidence in this migrant population, therefore, environmental factors must play a very important role in the etiology of type I diabetes.

Many candidate environmental factors have been proposed and studied, including virus and bacterial infections, childhood diet, standard of hygiene and vaccinations, chemicals, drugs, geographical latitude, etc. Among all environmental factors, viral infections and diets are believed to be more likely to trigger type I diabetes (Akerblom and Knip, 1998).

Viral Infections

Viral infections have been implicated in the etiology of Type I diabetes (Akerblom and Knip, 1998). There is epidemiological data in support of this idea. Rewers et al. (1987) conducted a survey in midwestern Poland area. They found that the incidence rates for type I diabetes in children aged 0-16 years had a seasonal variation ($P<0.001$), with the highest rate in the autumn-winter, which was consistent with increases in the likelihood of encountering pathogenic viruses in closed environments. Wagenknecht et al. (1991) analyzed 266 cases of type I diabetes obtained from the diabetes registry in Albama from 1979 to 1988. They found the temporary increased incidence of this disease in 1983 was concordant with the epidemic of coxsackievirus B5 that occurred in the same year. The authors suggested that this virus might play a role in the development of type I diabetes.

However, there are some other studies which showed conflicting results. Signs of an autoimmune attack upon the β cells of the pancreas can be detected years, not weeks or months, before the appearance of disease symptoms, while Rewers et al. (1987) and Wagenknecht et al. (1991) reported that type I diabetes epidemics occurred only months after peak incidences of viral infection. Buesa-Gomez et al. (1994) and Foy et al. (1994) used reverse transcription and a polymerase chain reaction (PCR) technique to investigate the pancreatic tissues from the diabetic patients. They failed to find any viruses such as mumps, rubella, polio, etc. Therefore, they suggested that the type I diabetes was not caused by the direct infection of the pancreatic β cells by viruses.

Diet

There are growing evidences suggesting that diet plays an important role in triggering type I diabetes. Case control studies showed that the incidence of type I diabetes was greatly decreased among the children who had been breast-fed to an older age (for breast-feeding duration ≥12 months), which suggested that the breast milk might have a protective effect on the development of this disease (Mayer et al., 1988). In rodent studies, animals on different diets have a significant difference in the incidence of type I diabetes. Cereal-based diets such as an NIH diet (diet formula in Appendix F) can cause higher diabetes incidence while hydrolyzed casein (HC)-based diet (diet formula in Appendix G) can protect against diabetes (Wang et al., 2000; Elliott and Martin, 1984, Elliott et al., 1988, Scott et al., 1985; Hoorfar et al., 1993). This idea was supported by the pooled data from eight experiments using the NIH diet and HC diet to feed the BBdp rats between 1983 and 1996 (Scott, 1996). At the age of 160 days, 63% of NIH-fed BBdp rats developed diabetes while the incidence in rats on HC diets was only 12-13%. The insulitis score of BBdp rats on HC diet (1.7) was much less than that on NIH diet (3.8). The age of onset of overt diabetes was delayed by 16 days by HC diet compared to NIH diet. Meanwhile, HC-fed rats maintained more normal pancreas histology (28% of islets infiltrated with immune cells in HC-fed rats compared to 77% in NIH-fed rats).

Scott et al. (1997) proposed that the outcome of diabetes by diets is closely related to the dose and timing in rodent feeding studies. They fed BBdp rats from weaning to 133 days with a protective HC-based diet, a diabetogenic NIH diet or a mixture of NIH:HC diet with the ratio either 3:7 or 7:3. The results showed that there was a dose response to the amount of NIH component in the diet that was associated with the diabetes incidence and severity of insulitis. The 100% NIH diet caused the highest diabetes incidence and the most severe insulitis. The 70% NIH diet caused a similar incidence but moderate insulitis, while the 30% NIH diet and HC diet caused low incidence and very mild insulitis. This research group also examined the effect of the time of introduction and duration of exposure to the diabetogenic diet on diabetes. In the timing study, BBdp rats were fed an HC diet or NIH diet from weaning at 23 days. At 50 days, diets were switched from HC to

NIH or from NIH to HC. In the duration study, animals were fed with HC diet until 100 days and then switched to the NIH diet. In the rats fed with HC from 23 days of age and then switched to NIH at 50 days, the onset time of diabetes was delayed. After switched to NIH diet, these rats experienced diabetes at the same rate and they had the similar ultimate incidence to those exclusively on NIH diet. Animals fed with NIH diet then switched to HC diet at 50 days had a lower diabetes incidence compared to those completely on NIH diet, and the insulitis score was similar to that of HC-fed rats. Animals on HC diet until 100 days had a lowest diabetes incidence and insulitis value compared to those on NIH diet during the whole study. These important findings suggested that the amount of diabetogens in the diets directly affected the outcome of type I diabetes. The dietary control of diabetogenesis appeared to be cumulative. It was not only restricted to early infancy in a triggering-like process, but the whole period from the beginning of puberty to late adolescence was also very important.

Many studies have been conducted to identify the diabetogens in diets. Dietary proteins have been studied extensively as a possible diabetogen. Milk proteins and wheat proteins are found to induce a higher incidence of type I diabetes, while casein results in lower diabetes incidence (Beppu et al, 1987; Martin et al, 1991; Savilahti et al, 1993). A bovine serum albumin (BSA) peptide in cow's milk, ABBOS, has been proposed as a possible trigger of this disease (Karjalainen et al., 1992; Robinson et al., 1993). However, it is still under dispute because of some conflicting results. For instance, Atkinson et al. (1993) found that there was lack of responsiveness of peripheral-blood mononuclear cells to BSA or ABBOS in type I diabetic patients or subjects with high risk for this disease. Paxson et al. (1997) compared the effect of a standard rodent diet which included 0.25% of dried cow's milk whey protein and a modified milk-free diet on the diabetes incidence in NOD mice. They found that the milk-free diet failed to decrease the diabetic incidence in NOD mice thus exhibiting no preventive effect.

Wheat has been identified as one of the most diabetogenic ingredients in all the rodent diets. It is one of the major components (33%) of the NIH diet, which is a rodent standard diet developed by the National Institutes of Health (Scott, 1996). Wheat may be responsible for the diabetogenic effect of the NIH diet. Scott (1996) and his colleagues fed

semi-purified diets to BBdp rats from weaning at 23 days of age to 162 days with exclusive protein sources including casein, wheat gluten, rapeseed flour, etc. The results showed that the wheat gluten-based diet resulted in the highest incidence (40%), while only 10-15% of rats on casein-based diet developed type I diabetes ($P<0.0001$). The severity of insulitis also increased significantly in the rats on the wheat gluten-based diet.

The diabetogenic potency of wheat is also confirmed by another study in which NOD mice were used as rodent models (Funda et al., 1999). In this study, two groups of 28 female NOD mice were fed with a standard rodent diet or a gluten-free diet from weaning at 21 days of age. Both of these two diets contained about 22.8% of protein. There was 2.5% of wheat protein in standard diet, while in gluten-free diet, wheat gluten was replaced by meat protein The results showed that a substantially lower diabetes incidence (15%) was achieved by gluten-free diet compared to a high incidence (64%) in standard diet in NOD mice at the age of 320 days ($P<0.0001$). Meanwhile, the onset time of diabetes was also significantly delayed in mice on a gluten-free diet (197 days vs 244 days). Histological examination showed a lower rate of islet abnormalities in wheat gluten-free group compared to standard group.

Recently, a blinded international trial was performed in three different countries under the same protocols to evaluate the effect of three diets on type I diabetes in both NOD mice and BBdp rats: a milk-free, wheat-based NTP-2000 diet (diet formula in Appendix E) and two casein-based diets with two different purified fractions from whole casein, named A^1-β-casein and A^2-β-casein. NTP-2000 is a certified rodent diet developed by the U.S. National Toxicology Program of the National Health Sciences. Wheat is a major component (37%) in this diet. The results in all these three locations showed that NTP-2000 diet caused the highest diabetes incidence in both of the animal models, which further proved the diabetogenic property of wheat (Beales et al., 2002).

Wheat-based diet induces type I diabetes in animals probably through changing two factors: the target β-cells and cytokine patterns in islets (Scott, 1996). Semiquantitative morphometric analyses of the pancreases showed that BBdp rats on NIH diet had 65% less islet area than those on HC-diet at 41 days of age (Scott et al., 1997). The mRNA expression of certain cytokines in pancreas was also different in BBdp rats on different

diets. There was a shift from Th2 type to Th1 type in the islets of NIH fed rats. For instance, at age 70 days, the expression of IFN-γ, a typical Th1 cytokine, was significantly higher in the pancreas of NIH-fed rats than that of HC-fed rats (Scott et al., 1997). MHC Class I molecules were also found highly expressed in β-cells from BBdp rats on wheat-based diet, which might be induced by the higher expression of IFN-γ (Scott, 1996).

All these studies strongly suggest that there must be some substances in wheat which are very important in changing target β-cells, altering cytokine patterns in islets, and promoting type I diabetes. Diabetogens in wheat have been reported to be extracted by 2:1 v/v mixture of chloroform and methanol (CM) by Coleman et al. (1990). In their study, natural-ingredient diet OG96 was treated with a 2:1 CM mixture and both the extract and remaining residue were dried under a hood. The CM extract and residue were then incorporated separately into the casein-based AIN-76 diet. The amount of extract or residue in one kilogram of diet was equivalent to a 25% (w/w) supplement of OG96 in AIN-76. NOD mice were fed from the weaning date with AIN-76, OG96, AIN-76 + 25% CM-insoluble residue of OG96 or AIN-76 + 25% equivalent CM-extract from OG96. The results showed that at 30 weeks of age, the diabetes incidence in these four groups were 17%, 64%, 30% and 75% respectively. The investigators suggested that the CM extraction can remove the diabetogens from wheat, which caused a reduced diabetes-promoting activity of wheat. The unknown substances in the CM extract which were responsible for the diabetogenic character of wheat must be lipoidal.

A CM mixture is widely used for extraction of lipids from animal and plant tissues. Sphingolipids are one of the classes of lipids that are soluble in mixture of CM (Christie, 1996). Sphingolipids, especially ceramides, have very important cell-regulation functions. They are also found to affect β-cell functions and induce β-cell apoptosis in cell culture studies (Sjorholm, 1995; Shimabukuro et al., 1998; Major et al., 1999).

Sphingolipids

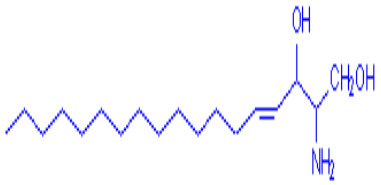
Structure

Sphingolipids are one class of complex lipids. All sphingolipids contain long-chain sphingoid bases. The most abundant base in animal tissues is sphingosine, which contains

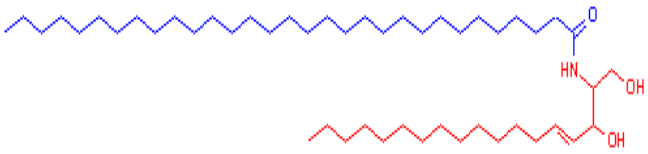
a C_{18} aliphatic chain, with hydroxyl groups in positions 1 and 3, an amine group in position 2, and a trans-double bond in position 4 (Figure 1). Ceramides are amides of fatty acids linked to the sphingoid base via an amide bond. Free ceramides are found in small amounts in plant and animal tissues, but generally they form the basic building blocks of the sphingolipids (Christie,1973). Sphingolipids usually have headgroups, which range from phosphodiesters to glycosides with simple to complex carbohydrate moieties (Schmelz and Merrill, 1998). For instance, sphingomyelin, a major component of the complex lipids in all animal tissues, consists of a ceramide unit linked to a phosphorylcholine at position 1.

The term "glycolipid" is used to describe any lipid classes containing a sugar residue. There are two main glycosphingolipids: uncharged cerebrosides and acidic gangliosides. Cerebrosides are the most widely occurring glycosphingolipids. They consist of the basic ceramide unit linked at position 1 by a glycosidic bond to oligosaccharide units, such as glucose or galactose. Cerebrosides are widely present in animal tissues and plants. Gangliosides contain basic ceramide bases and one or more sialic acid groups (NANA). They are found in many animal tissues but not in plants (Christie, 1992).

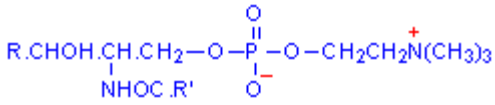
There are more than seventy different sphingoid base backbones that vary in alkyl chain length, degree of saturation, and position of the double bond. The fatty acids in sphingolipids also vary in chain length (usually 14-30 carbon atoms), the degree of unsaturation, and the presence or absence of hydroxyl group on α-carbon atom (Vesper et al., 1999). More than 300 headgroups are identified (Merrill et al. 1997a). These properties make sphingolipids the most structurally diverse class of lipids.



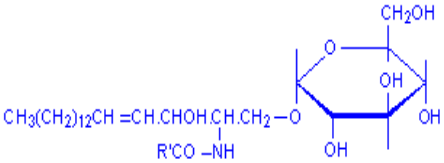
sphingosine



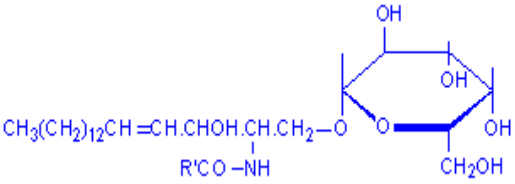
ceramide



sphingomyelin



glucosylceramide



galactosylceramide

Figure 1: Structures of sphingosine and sphingolipids

Sphingolipids in Foods

Sphingolipids are constituents of most foods, especially foods of mammalian origin, such as dairy products and meat products. For instance, the content of sphingolipids (estimation based on glycolipid content only) is 1326 μmol/kg in cheese, 1692 μmol/kg in cream, 160 μmol/kg in milk, and 530 μmol/kg in chicken. Sphingolipids also exist widely in many plant foods, such as wheat flour (Fujino and Ohnishi, 1983), rice (Fujino and Ohnishi, 1976), soybeans (Sullards et al., 2000), and fruits (Kim et al., 1997; Yamauchi et al., 2001), etc. In wheat flour, the sphingolipid content is about 576 μmol/kg. Since the yearly consumption of wheat is higher than any other foods, the sphingolipids from wheat consumed per capita is nearly the highest in all foods, reaching 38,016 μmol/year, which is close to the sum of sphingolipids consumption from all dairy products (Vesper et al., 1999).

Foods from mammalian origin have a wide spectrum of complex sphingolipids, including sphingomyelins, cerebrosides, and gangliosides, etc (Sang et al., 2002), while most of the sphingolipids in plants are present in the forms of cerebrosides. There are two types of cerebrosides: neutral cerebrosides and negatively charged phytoglycolipids. The neutral cerebrosides have a glycosyl or oligosaccharide unit at the primary hydroxyl group of sphinganine derivative. Phytoglycolipids are derivatives of ceramide-1-phosphate to which glycosylated inositols are bound via a phosphodiester linkage (Christie, 1982).

The sugar residues in plant cerebroside molecules are usually glucose and mannose. Glucosylceramide is the main basic structure used for the further β (1→4) linked mannosylations which results in series of di-, tri- and tetraglycosyl ceramides (Sperling and Heinz, 2003). This is the characteristic of wheat cerebroside molecules. In wheat grain, the primary glycosphingolipids are glucosylcerebroside (GlcCer). Diglycosylceramide (Man-GlcCer), triglycosylceramide (Man_2-GlcCer) and tetraglycosylceramide (Man_3-GlcCer) also exist in smaller amount (Fujino and Ohnishi, 1983).

Column chromatography is usually used to extract large quantities of cerebrosides from plants. In this technique, silica gel is used as the stationary phase and placed in a vertical glass column and the mobile phase, a liquid, is added to the top and flows down through the column. The extracted lipid mixture is applied to the top of the column. The liquid solvent (the eluent) is passed through the column by gravity or by the application of

air pressure. An equilibrium is established between the solute adsorbed on the silica gel and the eluting solvent flowing down through the column. Because the different components in the mixture have different interactions with the stationary and mobile phases, they will be carried along with the mobile phase to varying degrees and a separation will be achieved. The individual components, or elutants, are collected as the solvent drips from the bottom of the column. The neutral lipids are removed with chloroform, and then acetone is applied to remove glycolipids. Sphingolipids are in the acetone fraction. Glycosylsphingolipids are always obtained together with sterol glycosides, and sometimes even some phosphoglycolipids may also be present in this fraction. Phospholipids are removed by saponification, in which lipids are mixed with 0.4M potassium hydroxide (KOH) in methanol for 2h at 38^{o}C. Sterol glycosides and different sphingolipids are separated by consecutive thin layer chromatography (TLC) with different developing solvents (Christie, 1996).

Using these techniques, Fujino and Ohnishi (1983) obtained 100mg of ceramide, 580 mg of monoglycosylceramide, 17 mg of diglycosylceramide, 38 mg of triglycosylceramide and 4mg tetraglycosylceramdie from 3 kg of wheat grain. Since sphingolipid molecules lack a chromophore, it is hard to identify these molecules directly with ultraviolet (UV) detection. Evaporative light-scattering detector (ELSD), a mass detector, is now widely used with HPLC in lipid analysis. A light-scattering detection does not require the derivatization of sphingolipids. By using this detector, the solvent from column is evaporated in a stream of nitrogen gas, while all the nonvolatile solute particles form minute droplets and pass through a light beam which is reflected and refracted. The amount of scattered light is measured and has a relationship to the mass of the sample (Christie, 1985). With this technique, Sugawara and Miyazawa (1999) analyzed the glycolipids from edible plants. They found that ceramide monohexoside (CMH) is widely distributed in cereals, legumes, vegetables and fruits. In wheat flour, the content of CMH is about 21mg/100g.

Mass spectrometry (MS) is widely used for determination of structure of ceramide and cerebrosides (Yamauchi et al., 2001; Juang et al., 1996). The structure of wheat glucosylceramides (GlcCer) was determined by tandem mass spectrometry (MS) by

Sullard et al. (2000). The MS graph showed that wheat GlcCer has three major ceramides, sphingoid base d18:2$^{\Delta4,\Delta8}$ with fatty acid chain h16:0, sphingoid base d18:1$^{\Delta8}$ with fatty acid chain h16:0 and sphingoid base d18:2$^{\Delta4,\Delta8}$ with fatty acid chain h20:0 (d denotes a dihydroxy base with hydroxyl groups in 1 and 3 position in sphingoid base, Δ denotes the position of double bonds, h denotes an α-hydroxy group in fatty acids)

Metabolism of Sphingolipids

When dietary sphingomyelin or cerebroside is fed to rodents, not all of ingested sphingolipids are hydrolyzed and absorbed. Nilsson (1968) found that about 25% of administrated sphingomyelin with radiolabeled sphingosine was excreted in rat feces, of which 6-12% was the intact molecules, 80-90% was ceramide and 3-6% was free sphingosine. Nilsson (1969a) also reported that when cerebrosides were used to feed rats, 41-46% of cerebrosides with radiolabedled sphingosine were excreted in the feces, of which 40-75% were intact molecules and 25-60% were ceramides.

Nilsson (1968 & 1969a) found that sphingolipids were hydrolyzed mostly in the intestinal tract of rats. Schmelz et al. (1994) had the same observation in a mice study. The alkaline sphingomyelinase, an enzyme that hydrolyzes sphingomyelin, was first found in rat intestinal brush border (Nilsson, 1969b) and its distribution and characteristics in rat gastrointestinal tract were further studied (Duan et al., 1995). Both alkaline and neutral sphingomyelinase activity is found in human pancreatic juice (Nyberg et al., 1996; Chen et al., 1992). Meanwhile, the neutral sphingomyelinase is also found in human small intestines (Chen et al., 1992). Recently, the presence of ceramidase, an enzyme that breaks down ceramides, was also reported to be present in the intestinal contents of rats and humans (Duan et al., 2001).

The hydrolysis products of sphingolipids, such as sphingosine and ceramide, are rapidly taken up by intestinal cells (Vesper et al., 1999). Diet is believed to be one of the two major sources of the free sphingosine in cells (another source is the turnover of complex sphingolipids) (Merrill et al., 1997b). When radiolabeled sphingolipids were fed to rats or mice, some radiolabeled sphingosine, fatty acids, sugar and complex sphingolipids were found in blood and some other tissues, such as lymph, liver, etc

(Nilsson, 1968; Nilsson, 1969a; Schmelz et al., 1994). This suggests that the hydrolysis products of sphingolipids are transported through the mucosa into systemic circulation (Vesper et al., 1999). Some metabolites of sphingolipids are able to reincorporate into other complex sphingolipids, such as cerebroside and ganglioside (Vesper et al., 1999).

Possible Mechanisms for Triggering Type I Diabetes by Sphingolipids

Sphingolipids may be the diabetogens in wheat which trigger type I diabetes. There are at least two possible mechanisms for the effect of sphingolipids on the dysfunction of pancreatic β-cells: (1). Cell-regulation functions of sphingolipids and (2). Disturbance of gut immune system by sphingolipids.

Cell-Regulation Functions of Sphingolipids

Sphingolipids and their metabolites have been proven to play a very important role in cell regulation. These molecules function as second messengers inhibiting cell growth and inducing apoptosis in many types of cell lines (Okazaki et al.,1990; Fishbein et al., 1993; Hannun and Obeid, 1995).

Sjorholm (1995) used fetal Wistar rat islet to investigate the effect of ceramide on islet β-cells. The results showed that the treatment of exogenous cell-permeable ceramide to the β-cell culture medium caused a significant cytotoxic effect on β-cells with decreased β-cell proliferation and reduced insulin content in islets. This also occurred when cells were exposed to IL-1β. The addition of sphingomyelinase to the β cell culture to induce the production of ceramide by hydrolysis of sphingomyelin caused the same effect. The author concluded that ceramide, either exogenously delivered or endogenously generated by the hydrolysis of sphingomyelin, could mimic the cytotoxic effect of IL-1β and inhibit pancreatic β-cell insulin production. These findings were confirmed by another study performed by Major et al. (1999). In their study, ceramide was found to decrease agonist-induced insulin secretion in β-cells and have a time- and dose-dependent cytotoxic effect on β-cell cells.

Ceramide is found to induce apoptosis in many cell lines (Haimovitz-Friedman et al., 1994; Jarvis et al., 1994; Shimabukuro et al., 1998). When cells are treated with apoptosis-

promoting agents, such as TNF-α, FAS antigen activation or irradiation, ceramide is generated as the results of sphingomyelin hydrolysis (Dressler et al., 1992; Haimovitz-Friedman et al., 1994; Kolesnick et al., 1994). The exposure of cells to apoptosis-promoting agents or exogenous ceramide causes the fragmentation of genomic DNA resulting in the laddered patterns of oligonucleosomal fragments in agarose gel electrophoresis, which is a hallmark of apoptosis. For instance, when leukemia cells were treated with 5 μM synthesized cell-permeable ceramide for 3 hours, fragmentation was found in genomic DNA from cells. With the increases of ceramide concentration, DNA fragmentation also increased. Other amphiphilic lipids such as fatty acids did not cause DNA fragmentation, suggesting the specificity of actions of ceramide (Obeid et al., 1993).

Apoptosis is the main mode of β-cell death in type I diabetes (Eizirik and Darville, 2001; Mandrup-Poulsen, 2001). DNA is a target of cytokine actions in pancreatic β-cells and DNA fragmentation is an early event of β-cell apoptosis in type I diabetes (Rabinovitch et al., 1994). Ceramide is involved in β-cell apoptosis. Shimabukuro et al. (1998) found that in diabetic islets from Zucker Diabetic Fatty (ZDF) rats, an animal model of type II diabetes, the ceramide level was significantly higher than that in age-matched lean wild-type controls at 7 weeks, and this level was further increased at 14 weeks. To examine the effect of ceramide on β-cell apoptosis, pancreatic islets were isolated from prediabetic rats and were cultured with 15 μM ceramide or 50 μM fumonisin B_1, a ceramide synthesis inhibitor. The results showed that the ceramide treatment caused a two-fold increase in DNA fragmentation. In contrast, the addition of fumonisin B_1 almost completely prevented DNA fragmentation. These findings strongly suggested that the ceramide acted as an important mediator in apoptosis in these islets.

Ceramide may regulate β-cell apoptosis through activating certain proteins, such as protein kinase C (PKC) and protein phosphatase 2A (PP2A). When cells are treated with TNF-α, ceramide is generated to regulate the activity of PKC by PKCα dephosphorylation which consequently induces apoptosis (Lee et al., 2000). The activity of PP2A was found to exist in isolated beta (HIT-T15 or INS-1) cells. When the beta-cells were treated with exogenous ceramide, the insulin secretion was decreased and PP2A activity was increased in a ceramide concentration-dependent manner. It was suggested that ceramide exerted its

effects on β-cells via PP2A leading to an altered insulin secretion and decreased cell viability followed by beta cell apoptosis (Kowluru and Metz, 1997).

Disturbance of Gut Immune System by Sphingolipids

The basic property of the immune system is its ability to discriminate between self and non-self, also called immunologic tolerance. This property enables the immune system to protect the host from foreign antigens without reacting against itself. When the immune tolerance to self-antigens is broken, autoimmune disease such as type I diabetes develops (Elgert, 1996). There are two types of tolerance: central tolerance and peripheral tolerance. Central tolerance controls the induction of self-tolerance by the process of negative selection of self antigen-reactive T and B cells in the thymus/bone marrow, while peripheral tolerance controls tolerance to the peripheral antigens outside the thymus/bone marrow (Elgert, 1996). Oral tolerance, also called mucosal tolerance, is the main component of peripheral tolerance and refers to a systemic nonreactiviy to an orally administrated foreign antigen in the gut system. The induction of oral tolerance needs two exposures to the same antigen: a first tolerance-eliciting exposure and a second challenging exposure to the same antigen (Weiner, 1997).

A link between gut immunity and autoimmune diabetes has been suggested by Harrison and Honeyman (1999). The gut-associated immune system was thought as the primary target of all types of diet components. Since gut immune system comprises about two thirds of the total lymphoid tissue in human, it is expected that the disturbance of gut immune system by certain dietary constituents may have a detrimental effect on the whole body immune system, possibly through the release of immune cells or immune mediators produced in the gut system (Kolb and Pozzilli, 1999).

Physiological maturation of the gut immune system and the development of oral tolerance are affected by cytokines in the gut system (Goebel et al., 1999). Breastfeeding is found to have a protective effect on the onset of type I diabetes (Mayer et al., 1988), possibly because breast milk contains a lot of growth factors and cytokines such as TGF-α and M-CSF which are important for the maturation of intestinal mucosal tissues. The development of oral tolerance also requires colonization of gram-negative bacteria in the

gut early in life (Wasmuth and Kolb, 2000). NOD mice are found to have a higher diabetes incidence when they are maintained under germ-free environment compared to those under conventional conditions (Pozzilli et al., 1993). This is possibly due to the fact that hygienic environment delays colonization of gram-negative anaerobes in the gut (Kolb and Pozzilli, 1999). Recently, Gale (2002) proposed a hygiene hypothesis that the improved living conditions may contribute to the steady increase of type I diabetes, possibly through weakening the mucosal immune system.

It has been proposed that β-cell autoreactive lymphocytes in type I diabetes may belong to the gut-associated lymphocytes and may even originate from the gut mucosa (Vaarala, 1999). Hannine et al. (1993) examined a T-cell line propagated from pancreatic islets from an 8-year-old diabetic girl at the onset of diabetes and compared these cells' endothelial binding property to vascular endothelium in different body regions. Another T cell line was also developed from the peripheral blood mononuclear cells from that diabetic patient and these cells were used as controls. This study found that the control T cell lines did not show any preferential binding to the endothelium in normal pancreas, but showed an increased binding in diabetic pancreas. The pancreatic cells exhibited strong adherence to endothelium of mucosal lymphoid tissue and diabetic pancreas, but the binding of these cells to the endothelium of peripheral lymph node was weak. This study suggested lymphocytes derived from mucosal lymphoid tissue might be involved in the onset of type I diabetes and the interactions between lymphocytes and endothelial cells were important for the accumulation of autoreactive immune cells in the pancreas.

Homing of circulating lymphocytes from blood into gut-associated lymphoid tissue (GALT) or other lymph nodes is a complex process involving the interaction of lymphocyte cell adhesion molecules (CAM) with their endothelial cell ligands. The lymphocyte CAMs associated with tissue-selective migration are known as homing receptors. Their endothelial ligands which are expressed on the postcapillary venules of those tissues are called vascular addressins (Yang et al., 1996). Mucosal addressin cell adhesion molecule-1 (MAdCAM-1) is an adhesion molecule expressed by mucosa venules. These molecules selectively mediate the migration of circulating lymphocytes to GALT.

Lymphocyte adhesion molecules α4 and β7 are highly expressed by vessels in inflamed islets. These integrins are found to be important in the development of type I diabetes by mediating the migration of lymphocytes from blood into pancreatic islets in NOD mice. Treating NOD mice with mAb against α4 or β7 integrins can significantly block lymphocytic infiltration of islets and decrease diabetes incidence in these animals (Yang et al., 1994; Yang, 1997). Hanninen et al. (1996) found that most infiltrated lymphocytes in pancreas and islets in NOD mice expressed both α4 and β7. It was found that lymphocyte integrin α4β7 is a receptor of MAdCAM-1 (Berlin et al., 1993). MAdCAM-1 is a predominant addressin expressed on endothelial cells in and adjacent to the islets in NOD mice at early stages of insulitis (5-7 weeks). When the insulitis progresses, the number of vessels expressing MAdCAM-1 also increases (Yang et al., 1996). MAdCAM-1 is required in the recirculation and homing of lymphocytes before their accumulation in the pancreas. Research found that the blockage of MAdCAM-1 by weekly treatment of monoclonal antibody (mAb) from 3 weeks of age almost completely prevented diabetes ($P<0.01$) in NOD mice (Hanninen et al., 1998). It was suggested that the interaction of mucosal homing receptor α4β7 and its vascular addressin, MAdCAM-1, was a major adhesion pathway responsible for selective migration of lymphocytes from blood to inflammatory islets (Yang et al., 1996).

Animals or individuals that are genetically disposed to type I diabetes are found to have higher permeability of the intestinal epithelium. For instance, an increased gastric and small intestinal permeability is present in BBdp rats as early as 50 days of age which is before the development of both insulitis and clinical diabetes. The protective casein-based diet doesn't change this abnormality. Patients with type I diabetes who had the HLA-DQB 1*02 allele have been found to have higher intestinal permeability to lactulose and manitol (Kuitunen et al., 2002). This abnormality in intestinal permeability may facilitate the contact of certain food antigens with the mucosal immune system and cause intestinal inflammation. Hardin et al. (2002) examined inflammation in small intestine from BBdp rats by measuring the myeloperoxidase (MPO) activity, which is directly proportional to the numbers of neutrophils seen in histologic sections in an inflammation model. The results showed rats with 45 days of age displayed minimal intestinal inflammatory activity,

while after 70 days, there was a significant inflammatory activity throughout the small intestine. Mucosal inflammation may be also related to type I diabetes in humans. Savilahti et al (1999) reported that there was an increased inflammation in jejuna of type I diabetic patients. Compared to healthy controls, the diabetic patients expressed significantly more HLA-DR and HLA-DQ antigens in the surface of epithelium of the jejunum. These patients also had significantly more lymphocytes with α4β7 integrin, suggesting more T and B memory cells have accumulated in the intestinal mucosa.

All these finding suggest that gut immune system is involved in the pathogenesis of type I diabetes and the lymphocytes infiltrating into pancreatic islets are very likely derived from GALT. Disturbance of gut immune system may play a key role in the onset of type I diabetes. Sphingolipids may be involved in the breakdown of oral tolerance in diabetes susceptible individuals and may cause the destabilization of gut immune system, thus inducing the onset of type I diabetes.

CD1d molecules, which are MHC class I-like cell surface glycoproteins, have been reported to be expressed by intestinal epithelial cells in humans, rats and mice (Somnay-Wadgaonkar et al., 1998). These molecules are responsible for the presentation of mucosal glycolipid antigens to a specialized T-cell subset, known as natural killer or NK T cells (Wal et al., 2003; Kawano T, 1997). NKT cells are characterized by co-expression of NK cell receptors and semi-invariant T cell receptors (TCR), and they are key effector cells in innate immune responses. When activated, NKT cells produce high levels of IFN-γ and IL-4 and affect the immune response to antoantigens and tumors (Nishimura et al., 2000). Human NK T cells usually express a TCR consisting of Vα24 chain paired with Vβ11 chain (Naidenko et al., 2000). Certain glycosylceramides, such as α-glucosylceramide and α-galactosylceramide, are identified as ligands recognized by Vα24Vβ11NKT cells in a CD1d-mediated manner (Nieda et al., 1999). Nishimur et al. (2000) reported that *in vivo* administration by injection of glycosylceramide to C57BL/6 mice resulted in an increased NK activity in spleen cells. This activity was mediated by NKT cells since this effect was absent in NKT-deficient cells. The activation of NKT cells subsequently induced a rapid and substantial production of cytokines such as IFN-γ and IL-4. NKT cells also activated a

variety of cells of the innate and adaptive immune systems including CD4 T cells, CD8 T cells, B cells and macrophages, etc.

In the small intestine and Peyer's patches in NOD mice at age of 70-90 days, mRNA expression of some inflammatory cytokines such as IFN-γ, IL-10, and TNF-α were found significantly higher in wheat-fed mice than in casein-fed mice (Flohe et al., 2003). This change in cytokine patterns might be caused by glycosphingolipids in the wheat-based diet. Since not all the ingested wheat sphingolipids are hydrolyzed and absorbed in the intestine, some of the intact molecules such as glucosylceramides may be presented by intestinal epithelial cells to NKT cells as foreign antigens. This process is more likely to occur in genetic susceptible individuals with higher intestinal permeability. Activation of NKT cells then induces a strong production of cytokines such as IFN-γ. Recently, it was reported that frequency of Vα24Vβ11NKT cells in the peripheral blood of type I diabetic patients was significantly higher than that in the healthy controls (Oikawa et al., 2002). The activation of NKT cells by sphingolipids also induces the activation of other immune cells such as T cell and B cells. Some of these activated lymphocytes may migrate into pancreatic islets through α4β7/MAdCAM-1 interaction and the lymphocytes with autoreactive properties may attack β-cells and cause β-cell destruction.

Summary

From animal studies, diet factors play a very important role in the development of type I diabetes, with wheat-based diets causing the highest diabetes incidence compared to all other diets (Scott, 1996). It was suggested that the diabetogens in wheat could be extracted by chloroform-methanol mixture (Coleman et al., 1990). Sphingolipids are one class of complex lipids existing in wheat and can be extracted by chloroform-methanol mixture. There is evidence showing that sphingolipids have important functions on the regulation of pancreatic β-cell apoptosis (Sjorholm, 1995), and they may also cause the alteration of the gut immunity (Nieda et al., 1999), which is closely related to type I diabetes. Studies need to be conducted to examine the effect of wheat sphingolipids on type I diabetes, from both animal feeding studies and cell culture studies.

Chapter 4: Materials and Methods

Animals

Male and female BBdp rats were obtained from the Animal Resources Division of Health Canada. The animals were maintained in laminar flow protected cages under specific pathogen-free conditions. The mean incidence of diabetes in BBdp rats from this colony fed a standard cereal-based diet (Rao, 1996) has remained constant over the past 5 years at 65.3 ± 14.9% (mean ± SD). This colony was directly descended from the original diabetic rats discovered at BioBreeding laboratories near Ottawa in 1974 and transferred to Health Canada in 1977. The colony is not completely inbred, but has remained a closed colony for the past 25 years and recent genotyping for selected markers indicates the animals are ~ 80% identical at the DNA level. The colony was anitibody-free with respect to Sendai virus, pneumonia virus of mice, rat caronavirus/sialodacryoadenitis virus, Kilham rat virus, and Toolan's H-1 virus.

Wheat Gluten

Wheat gluten was purchased from ICN Biomedicals, Inc (Cleveland, Ohio). It contained 75-80% of protein.

Chemicals

All the chemicals used in this study were at least analytical grade. In HPLC analysis, all the solvents for mobile phase were HPLC grade. Water used in HPLC analysis was nanopure water from ultrapure water system (Barnstead/Thermolyne, Nanopure Infinity^{TM}).

Extraction of Sphingolipid-Enriched Lipid Fraction

Isolation of Total Lipids

Wheat gluten (6.5kg) was extracted with three volumes (19.5L) of chloroform-methanol mixture (2:1) for 24 hours with occasionally shaking. After centrifugation at 3,500 rpm for 20 minutes, the insoluble wheat gluten residue was collected and extracted

again with three volumes of chloroform-methanol mixture. The supernatant from two extractions was pooled and subjected to Folch wash (Folch et al., 1957). Briefly, the volume of supernatant was measured and 25% of the total volume of 0.88% KCl solution was added to remove the water-soluble non-lipid contamination. The upper water layer was removed. Sodium sulphate was added to the organic layer to remove the trace water. After filtration, the organic layer was dried using a rotary evaporator (Buchi, Switzerland) to remove all the organic solvent. The total lipid was collected and weighed. The chloroform-methanol insoluble gluten residue was spread in a tray under the hood for three weeks to evaporate the remaining organic solvent.

Fractionation of Total Lipid

The total lipids were dissolved in a small volume of chloroform (about 10ml for 20g lipids) and applied to a silica gel column. Six hundred grams of silica gel (70-230 mesh, average pore size 60 A; Sigma, Cat. No. S-2509) was placed in a 7 x 40cm glass column. Twenty grams of extracted lipid was applied to the top of silica gel at one time. The lipids were sequentially eluted with 10L chloroform and then 15L acetone. The acetone fraction was collected and the organic solvent was removed using a rotary evaporator.

Saponification of Lipids

The lipid from the acetone fraction was dissolved in 250ml of chloroform. Then 250ml of 0.4M KOH in methanol was added and mixed well. The mixture was kept at 37^{o}C water bath for 2 hours. After saponification, the mixture was filtered to remove the formed precipitate. The volume of filtrate was measured and then transferred into a preparatory funnel. One quarter of the total volume of 0.88% KCl solution was added to remove the water-soluble substances after saponification. Sodium sulfate was added to the alkaline-stable lipid portion to remove water. The organic solvent was then removed by a rotary evaporator. The dried lipid portion was collected and weighed. This was the sphingolipid-enriched lipid fraction which was used in the animal feeding study.

Proximate Analysis of Wheat Gluten before and after Chloroform-Methanol Extraction

Determination of Crude Protein

AOAC method 2.057 (AOAC, 1980), also called the Kjeldahl method, was used to determine the crude protein of the wheat gluten samples. A slight modification was made according to the instructions of the Kjeldahl instruments. The block digester unit was a Buchi Model No. 430 (Switzerland). The automatic steam distillation and titration unit was a Buchi Model No. 339. The crude protein content in wheat gluten was determined by multiplying the nitrogen content of the samples by 5.8 (Kies et al., 1978).

Determination of Crude Fat

AOAC method 7.056 (AOAC, 1980) was used to determine the crude fat in wheat gluten with modifications according to the instructions in the manual of the Soxtec fat extractor (Soxtec system HT6, Tecator AB, Sweden, Part No. 1000-1590). The method is based on the weight losses of samples caused by the circulating extraction with petroleum ether for 1.5 hrs, or the weight gain of the fat collected in containers after ether extraction.

Determination of Moisture

AOAC 7.007 (AOAC, 1980) was used to determine the moisture content in wheat gluten by drying the samples in aluminum pans of known weight in a Brabender oven (Hacksensack N. J., Model No. 692) at 130^{o}C for 3 hours.

Determination of Ash

AOAC 7.009 (AOAC, 1980) was used to determine the ash content in wheat gluten. Samples were weighed into porcelain crucibles and placed in a muffle furnace (Fisher, Model No. 495) at 600 ^{o}C for 2 hours.

Determination of Carbohydrate

The carbohydrate was determined by subtracting the percentage of crude protein, crude fat, moisture and ash from 100.

Detection of Lipids by Thin Layer Chromatography (TLC)

The TLC methods used in this study were those described by Robyt and White (1987) with a slight modification. Lipids were dissolved in 2:1 v/v chloroform-methanol mixture. Developing solvent was chloroform-methanol-water (65:16:2). TLC plates were obtained from Fisher (Silica Gel 60 F_{254}, 10x20cm; Cat. No. M5729-6).

Detection of Glycolipids

A spray reagent was prepared as follows: Orcinol (200mg) was dissolved in 100ml of sulfuric acid-water (3:1) and stored in the dark. 100µg of total lipid, 100µg of sphingolipid fraction and 5µg of a glucosylceramide standard (Sigma, St. Louis, USA) were applied to a TLC plate. After separation, the TLC plate was sprayed with the orcinol-sulfuric acid reagent until moist and then heated at 100^{o}C for 5min.

Detection of Sphingolipids

Two spray reagents were prepared for detection of sphingolipids. Reagent 1: 5ml of Clorox, 50ml of benzene, and 5ml of glacial acetic acid were mixed together. Reagent 2: 0.5g benzidene and one small crystal of potassium iodide were dissolved in 50ml of 50% ethanol and the solution was filtered. 500µg of total lipid, 500µg of sphingolipid fraction and 5µg of a glucosylceramide standard were applied to a TLC plate. After separation, the TLC plate was sprayed with reagent 1 and air-dried in the hood. Then the plate was sprayed with reagent 2.

Detection of Phospholipids

500µg of total lipid and 500µg of sphingolipid fraction were applied to a TLC plate. After separation, the TLC plate was lightly sprayed with molybdenum blue spray reagent purchased from Sigma (Cat. No. M3389).

Detection of Glucosylceramide by HPLC

Glucosylceramide in total lipids and sphingolipid-enriched fraction was analyzed by HPLC (Waters Assoc., MA, USA) with an evaporative light-scattering detector (ELSD) (Varex, MD, USA). The HPLC system consisted of a Waters Separations Module 2690 controller with dual pumps and Millennium data acquisition controller with analysis software. Nitrogen was used as a nublizing gas in ELSD. The method used was described by Sugawara and Miyazawa (1999) with a slight modification. HPLC-ELSD conditions were as follows:

HPLC Conditions:

Column: LiChrosher Si 60 (5μm, 125 x 4 mm i.d., Varian, PN 0114-125 x 040)

Guard Column: LiChrospher 5μm Si 60A, 4.6 mm, Varian

Flow Rate of Mobile Phase: 1ml/min

Injection Volume: 20 μl

Concentration of Samples: 10 mg/ml

Mobile Phase: A gradient system was used which consisted of chloroform and methanol/water (95:5, v/v)

Table 1. HPLC conditions for analysis of glucosylceramide in lipids

Time (min)	A(%)	B(%)
0	99	1
15	75	25
20	10	90
25	10	90
30	99	1

Solvent: (A) chloroform; (B) methanol/water (95:5, v/v)

ELSD Conditions:

Evaporation temperature: 60 ^{o}C

Pressure of nitrogen: 10 psi (40ml/min)

Detection of Glucosylceramide by Mass Spectrometry (MS)

Glucosylceramide was purified from sphingolipid-enriched lipid fraction using TLC plates (Silica Gel 60 F_{254}, 10x20cm; Cat. No. M5729-6). About 100mg of sphingolipid-enriched lipid fraction was dissolved in 2:1 (v/v) chloroform-methanol mixture and applied to one TLC plate. The developing solvent was chloroform-methanol-water (65:16:2 v/v/v). After separation, TLC plate was visualized under UV light (VWR, Model UVGL-25). The band corresponding to glucosylceramide was scraped and extracted by chloroform-methanol (2:1 v/v). After filtration, the filtrate was collected and dried under the flow of nitrogen. The purity of glucosylceramide was checked by TLC with chloroform-methanol-water (65:16:2 v/v/v) as developing solvent. The TLC plate was sprayed with 50% H_2SO_4 and charred at 100^{o}C for 5 minutes. The purity of wheat glucosylceramide was confirmed by HPLC under the same conditions as mentioned above. The purified glucosylceramide was analyzed by mass spectrometry (MS) using a JEOL JMS HX110 Dual Focusing Mass Spectrometer. The matrix used for positive ion fast atom bombardment (FAB) was 3-nitrobenzyl alcohol saturated with NaI (NBA/Na). The calibration standard was PEG (Polyethyleneglycol). A small amount of glucosylceramide (~ 1µg) was mixed with ~ 1µl of the matrix on a stainless-steel FAB probe tip. The FAB experiments were done under the following conditions:

Emission Current - 5 mA (milliamps)

Accelerating Voltage - 70 V (volts)

Ionizing voltage - 10 kV (kilovolts)

EF-Linear scan, scanning a 60 dalton region of 715 to 830

BBdp Rats Feeding Study

The animal feeding study was performed by Dr. Fraser Scott and his colleagues in Nutrition Research Division of Health Canada in Ottawa, Canada. Five animal diets were used in this study (diet formulas in Appendix E):

1. Group A: hydrolyzed casein (HC) based diet. In this diet, casein was used as sole amino acid source. This diet contained 53.0% of corn starch, 12.0% of sucrose,

18.0% of casein hydrolyzate (pancreas S enzymatic hydrolyzate, Redstar Bioproducts, Mississauga, Ontario), 7.0% of soybean oil, 5.0% of cellulose-type fiber, 3.5% of AIN-93G mineral mix, 1.0% of AIN-93G vitamin mix, 0.2% of choline bitartrate, and 0.3% of L-cystine.

2. Group B: National Toxicology Program 2000 (NTP-2000) diet. The NTP-2000 diet (Zeigler Bros., Gardners, PA) is an open formula, nonpurified diet for rodents developed by the U.S. National Toxicology Program of the National Institute of Environmental Health Sciences. This diet does not contain any milk protein. It is mainly plant-based with wheat as the major components (37%), followed by corn, soybean meal, alfalfa meal, oat hulls, fish meal and cellulose. The diet contains approximately 14.6% of protein, 8.2% of fat, 9.9% of crude fiber, 52% of carbohydrate, 10.7% of moisture; the remainder is native and added micronutrients. The NTP-2000 diet used in this study was irradiated, and contained low levels of chemical and microbial contaminants.
3. Group C: wheat gluten (WG) based diet. In this diet, wheat gluten was used as sole protein source. This diet contained 23.4% of wheat gluten, 49.0% of corn starch, 12.0% of sucrose, 6.1% of soy oil, 3.92% of cellulose-type fiber, 3.5% of AIN-93G mineral mix, 1.0% of AIN-93G vitamin mix, 0.2% of choline bitartrate, 0.3% of L-cystine, 0.5% of L-Lysine, and 0.08% of L-threonine.
4. Group D: wheat gluten based, sphingolipid-free (WGSLF) diet. In this diet, wheat gluten after chloroform-methanol treatment was used as protein source. This diet contained 23.6% of wheat gluten after chloroform-methanol extraction, 48.1% of corn starch, 12.0% of sucrose, 6.8% of soy oil, 3.92% of cellulose-type fiber, 3.5% of AIN-93G mineral mix, 1.0% of AIN-93G vitamin mix, 0.2% of choline bitartrate, 0.3% of L-cystine, 0.5% of L-Lysine, and 0.08% of L-threonine. To make the diet more palatable, pellets were coated with a sugar-based, artificial maple syrup-flavored liquid.
5. Group E: hydrolyzed casein plus a sphingolipid-enriched lipid fraction (HC + SL) diet. The sphingolipid-enriched fraction was extracted from wheat gluten as used in diet 4. It was suspended in soy oil and mixed with other components in HC diet to

make this diet. This diet contained 53.0% of corn starch, 12.0% of sucrose, 18.0% of casein hydrolyzate (pancreas S enzymatic hydrolyzate, Redstar Bioproducts, Mississauga, Ontario), 3.4% of soybean oil, 3.6% of soy oil containing sphingolipid-enriched fraction from wheat gluten, 5.0% of cellulose-type fiber, 3.5% of AIN-93G mineral mix, 1.0% of AIN-93G vitamin mix, 0.2% of choline bitartrate, and 0.3% of L-cystine.

BBdp rats were randomly assigned to one of the five diets after weaning at 23 days of age. Twenty rats were assigned to each diet. They were maintained in individual stainless steel cages and housed in a specific pathogen-free environment. Water and food were available ad libitum. Body weights were obtained once per week after diet treatment.

After 50 days of age, rats were tested twice each week for glucose in the urine using Testape (Lily, Toronto, Ontario). Those with a value of ≥2+ were fasted overnight and blood glucose in tail blood was measured in the next morning with a glucometer. Animals with blood glucose concentration ≥11.1 mmol/l were considered diabetic and were killed by exsanguinations under anaesthesia with 3% of halothane in oxygen. Non-diabetic animals were sacrificed at the end of the 125-day feeding study. The incidence of type I diabetes was calculated for each diet group. Pancreas and jejunum tissues from all the rats were saved for our follow-up studies.

Statistics

Survival analyses were carried out using Kaplan-Meier and log-rank tests. Data of diabetes incidence was analyzed using Fisher's Exact Test (two-tailed). The effect of diet on body weight gain was analyzed by two-way Analysis of Variance (ANOVA), and the mean of body weight gain was compared by Scheffe multiple comparison. Data of pancreas weight and diabetes onset age were analyzed by one-way ANOVA, and the differences between means were determined by Turkey-Kramer multiple comparison test. Differences were considered statistically significant if the p value was equal to or less than 0.05.

Chapter 5: Results and Discussion

Lipid Extraction:

Chloroform-methanol (2:1) was used to extract total lipid from wheat gluten. The extraction was performed twice to achieve more complete extraction. Altogether 152.5 g of total lipids were obtained from 6.5 kg of wheat gluten. The yield was about 2.4% of total weight. After fractionation by column chromatography, the recovered lipids in acetone fraction were 53.9 g, which was 35.3% of the total lipids. Fujino and Ohnishi (1983) used methanol to elute glycolipids from column. In our study, we used acetone instead of methanol to decrease the contamination of phospholipids, since methanol can elute most of the phospholipids. After saponification with 0.4M KOH in methanol, 40.4 g of the sphingolipid-enriched lipid fraction was recovered. This fraction was 26.5% of the total lipids, or 0.62% of the wheat gluten.

Proximate Analysis

The results of proximate analysis are shown in Table 2. After chloroform-methanol extraction, the content of crude protein, moisture and ash in wheat gluten were still similar to those in wheat gluten before chloroform-methanol extraction, while the content of crude fat decreased substantially. About 80% of crude fat was removed from wheat gluten after chloroform-methanol extraction. These results suggested that chloroform-methanol mixture is a very good solvent for lipid extraction. It can extract most of lipids without changing the content of other components such as protein, moisture and ash. There was still 20% of fat remaining in wheat gluten after extraction. Additional extractions with chloroform-methanol could be applied to remove more fat from wheat gluten, or alternative solvents could be used.

Table 2. Proximate analysis of wheat gluten before/after chloroform-methanol extraction

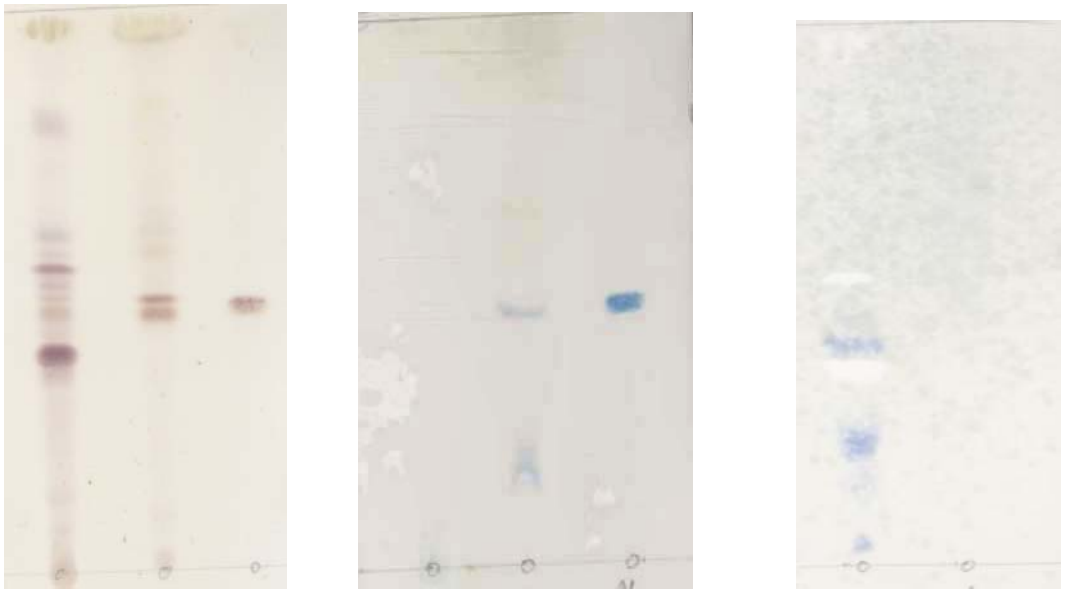
Items	Wheat gluten (%)	Wheat gluten after CM treatment (%)	Adjusted values for wheat gluten after CM treatment* (%)
Crude fat	0.934	0.198	0.193
Crude protein	76.80	76.28	74.49
Moisture	6.22	6.64	6.48
Ash	0.94	1.05	1.03
Carbohydrate	15.11	15.83	17.81

* Values were adjusted by multiplying by (6500-152.5)/6500 because of removal of 152.5g of total lipids from 6,500g of wheat gluten.

Detection of Lipids by TLC

TLC graphs for glycolipids, sphingolipids and phospholipids are shown in Figure 2. For glycolipids, only two main bands were found in sphingolipid-enriched lipid fraction with one band with R_f value similar to that of the standard glucosylceramide. However, in the total lipids, there were many glycolipid bands. When the same amount of sample was applied to TLC plates for sphingolipid detection, the band intensity of sphingolipids was much higher in sphingolipid-enriched fraction than in the total lipids, in which sphingolipids were barely detectable. There were two major bands corresponding to phospholipids in total lipids, while in sphingolipid-enriched lipid fraction, there was no phospholipid band at all. Sugawara and Miyazawa (1999) reported that after saponification of a wheat lipid extract, only two major glycolipids, sterol glucoside and glucosylceramide, were recovered. The mild alkaline condition in saponification can hydrolyze O-acyl linkage, while N-acyl linkage is stable under this condition (Sugawara and Miyazawa,

1999). Therefore, sphingolipids such as glucosylceramide which contain N-acyl linkage are recovered after saponification, while triglycerides which contain O-acyl linkage are all removed. Since sterol glucoside does not contain O-acyl linkage in its structure, it was also recovered after saponification.



1: Total lipids of wheat gluten
2: Sphingolipid-enriched lipid fraction from wheat gluten
3: Glucosylceramide standard (Sigma)

Figure 2. TLC analyses of glycolipids, sphingolipids and phospholipids in total lipids and sphingolipid-enriched lipid fraction from wheat gluten

Detection of glucosylceramide by HPLC

The HPLC graphs of total lipids and sphingolipid-enriched lipid fraction are shown in Figure 3 and 4 respectively. After fractionation and saponification, three components (peaks with retention time 9.2, 10.9 and 11.2 minutes) were enriched, while some other components, for instance, those peaks with retention time 5.5, 7.3 and 13.4 minutes, were removed. The peak with retention time 10.9min was identified as glucosylceramide by MS analysis. In total lipids, the area of glucosylceramide peak was 38930, while in sphingolipid-enriched lipid fraction, the area of this peak was 209076 (with the same concentration and same injection volume). The content of glucosylceramide in sphingolipid-enriched fraction was approximately 5.4-fold higher than that in total lipids. From these two graphs, some components with low polarity (shorter retention time), probably some neutral lipids which were not removed by enough chloroform in fractionation step, were also recovered after saponification. If these neutral lipids, for instance, sterols, do not contain O-acyl linkage in their structure, they are also stable under mild alkaline conditions.

The glucosylceramide standard used in HPLC analysis was isolated from human (Gaucher's) spleen. In the structure of most mammalian sphingolipids, the ceramide backbones contain mainly trans-4-sphingenine ($d18:1^{\Delta 4}$) and smaller amounts of sphinganine (d18:0) and 4-hydroxysphinganine (t18:0), while Δ-8 double bond in sphingoid base or α-hydroxy group in fatty acids chain only exists in very few cases (d means dihydroxy sphingoid base with hydroxyl groups in position 1 and 3, while t means trihydroxy sphingoid base with an additional hydroxyl group in a position other than 1 and 3; Δ indicates the position of double bonds). However, in the structure of most plant sphingolipids, the sphingoid bases include 8-sphingenine ($d18:1^{\Delta 8}$), 4,8-sphingadienine ($d18:2^{\Delta 4,\Delta 8}$) and 4-hydroxy-8-sphingenine ($t18:1^{\Delta 8}$) (Lynch, 1993). In wheat glucosylceramide, it was reported that there were three major sphingoid bases: $d18:2^{\Delta 4,\Delta 8}$, $d18:1^{\Delta 8}$ and $d18:2^{\Delta 4,\Delta 8}$ with fatty acids chain containing α-hydroxyl group (Sullards et al, 2000).

In our study, the difference in retention time of Gaucher's spleen glucosylceramide (9.7min) and wheat glucosylceramide (10.9min) in HPLC analysis was due to the structural differences in these two glucosylceramides. The presence of α-hydroxyl group in fatty acid chain in wheat glucosylceramide structure may result in an increased polarity compared to the Gaucher's spleen glucosylceramide, which lacks of α-hydroxyl group in fatty acid chain in its structure. Since a normal-phase column was used in HPLC analysis with increased percentage of methanol in chloroform as mobile phase, the component with higher polarity has a longer retention time. The difference of retention time of glucosylceramides from different sources was also observed by Sugawara and Miyazawa (1999).

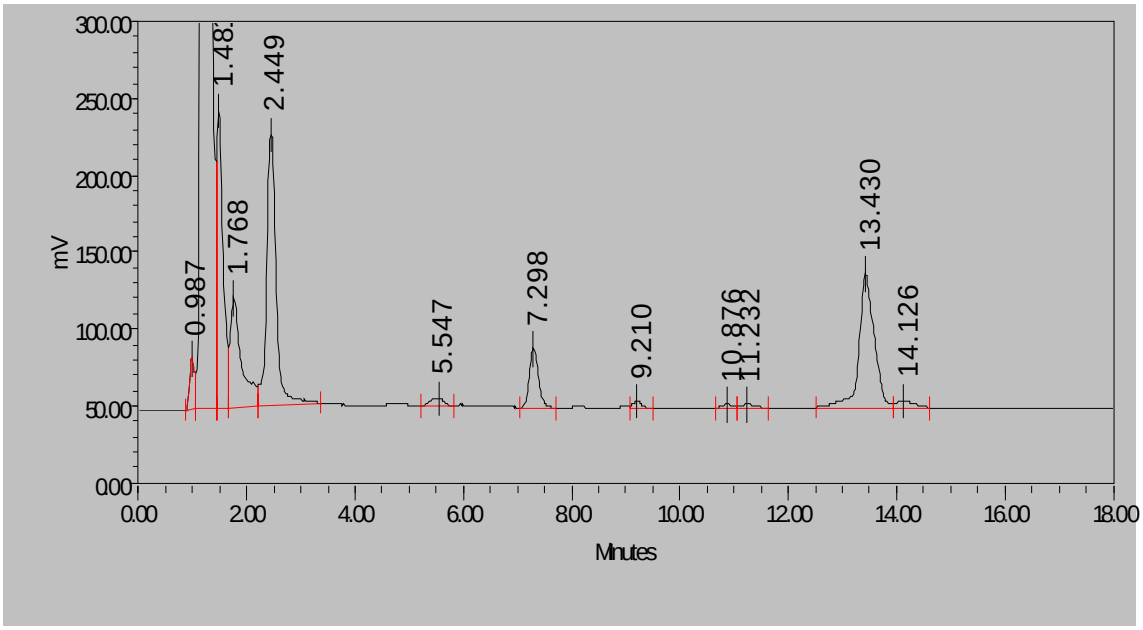


Figure 3. HPLC chromatograph of total lipids

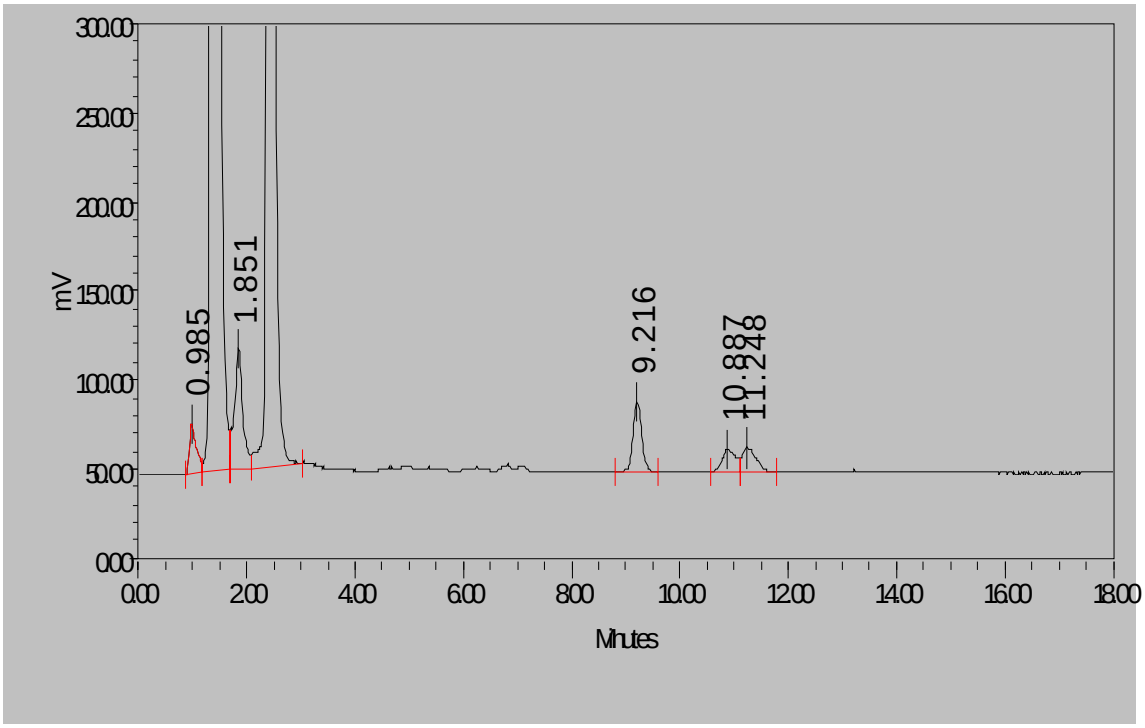


Figure 4. HPLC chromatograph of sphingolipid-enriched lipid fraction

Detection of Glucosylceramide by Mass Spectrometry (MS)

Wheat glucosylceramide was purified from sphingolipid-enriched lipid fraction using TLC plates under the irradiation of UV light and the purity was checked by TLC and HPLC. In TLC plate, only one band was shown after the plate was sprayed with 50% H_2SO_4, which suggested that a pure form of wheat glucosylceramide was isolated from sphingolipid-enriched lipid fraction. The HPLC graph of purified wheat glucosylceramide is shown in Figure 5. Only one major peak was found with the retention time at 10.9 minutes, which further indicated the high purity of wheat glucosylceramide.

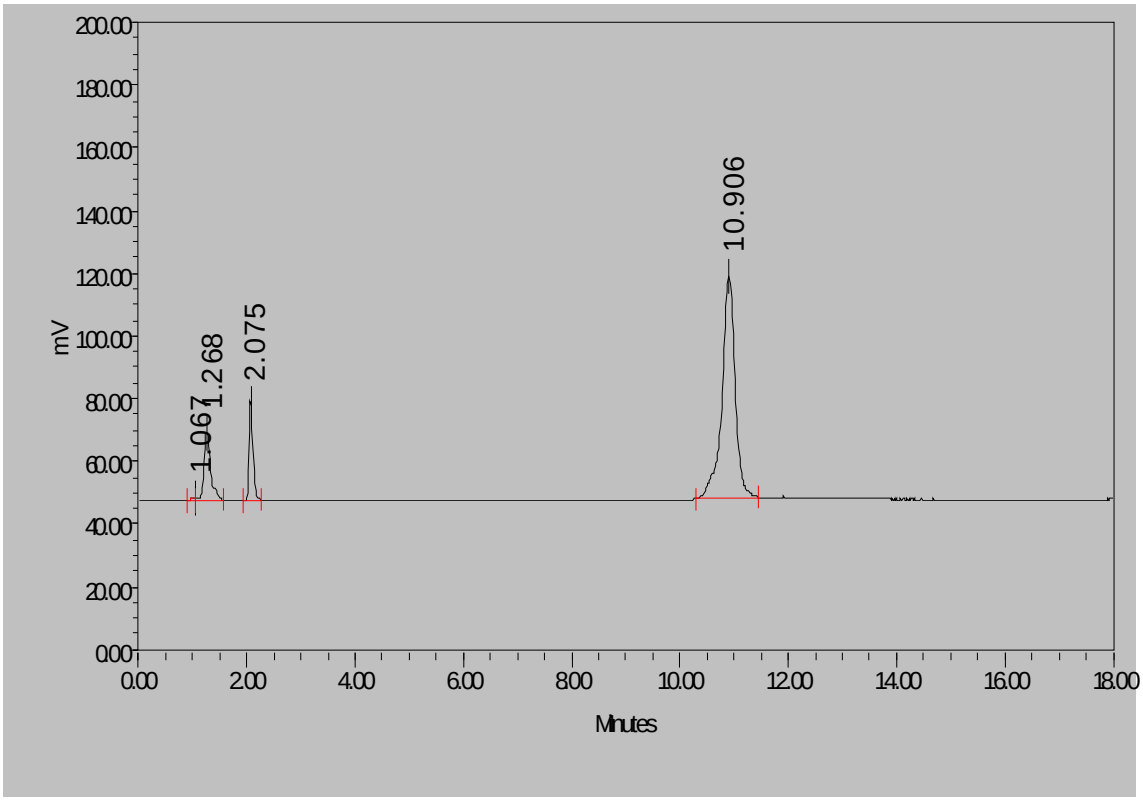


Figure 5. HPLC chromatograph of wheat glucosylceramide

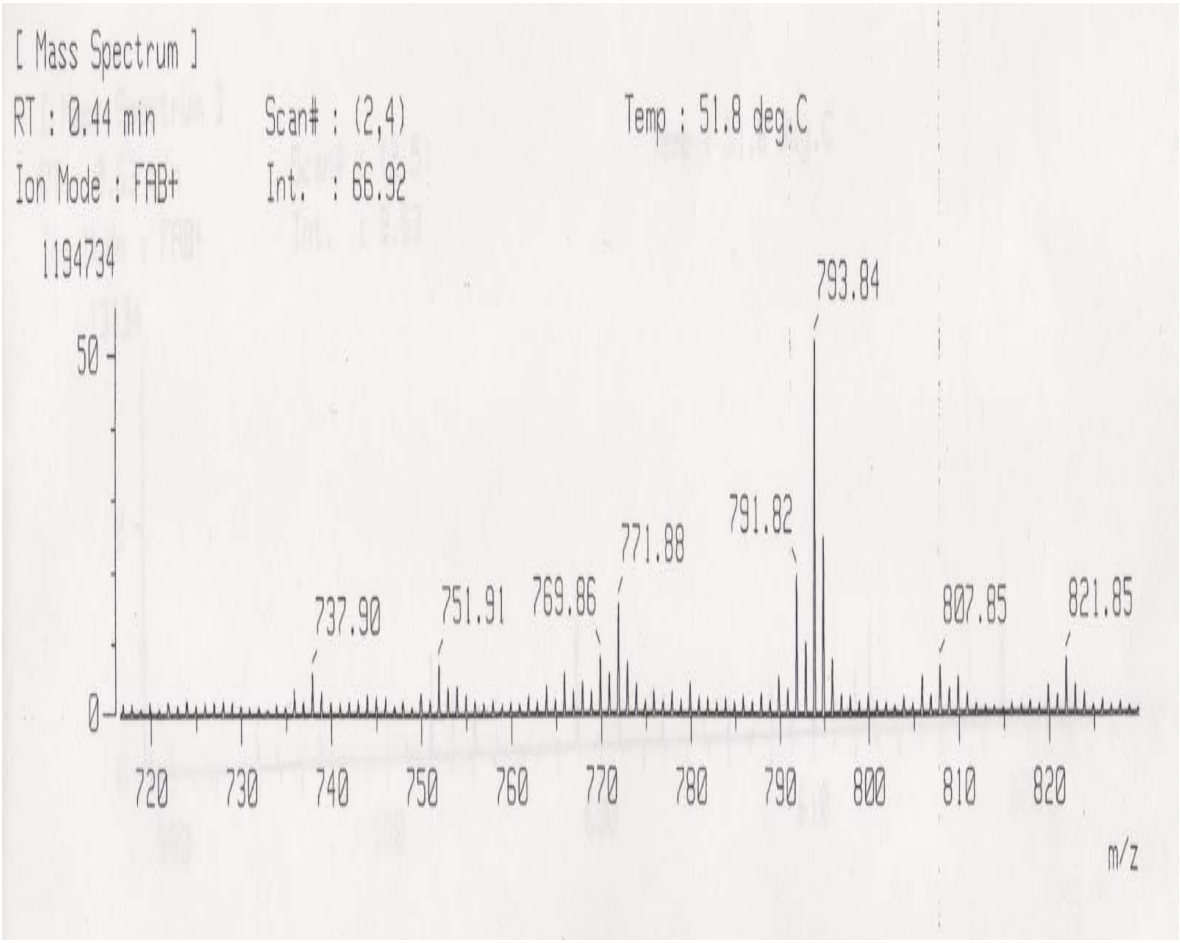


Figure 6. Mass spectrum of wheat glucosylceramide

The Mass Spectrum of glucosylceramide is shown in Figure 6. A major peak was found with $[M+Na]^+$ m/z (mass/charge) of 793.84, which suggested that the molecular weight of glucosylceramide was about 771 (793.84 minuses 22.98, which is the atomic weight of Na). The empirical molecular formula was $C_{44}H_{85}NO_9$ with very small error (error = +0.7ppm). Two research groups, Sullards et al (2000) and Sugawara and Miyazawa (1999), successfully isolated glucosylceramide from wheat flour and used mass spectrometry to analyze its structure. Sugawa and Miyazawa (1999) used electrospray ionization (ESI) and they found two major peaks in the mass spectra of wheat glucosylceramide with $[M+H]^+$ at m/z 716 and 772 respectively, suggesting two major structures of wheat glucosylceramide with molecular weight of 715 and 771 respectively. Sullards et al. (2000) used low-resolution, high-resolution and tandem mass spectrometry to examine the detailed structure of wheat glucosylceramide. They used positive ion fast atom bombardment (FAB) for ionization and LiI was used to increase the ionization. In their study, they found three major peaks in the mass spectrum with $[M+Li]^+$ at m/z 720, 722 and 776, suggesting three structures of wheat glucosylceramide with molecular weight of 713, 715 and 769 respectively. The MS-MS (MS^2) further demonstrated these three structures of wheat glucosylceramide: a sphingoid base $d18:2^{\Delta4,\Delta8}$ with fatty acid chain h16:0, a sphingoid base $d18:1^{\Delta8}$ with fatty acid chain h16:0 and a sphingoid base $d18:2^{\Delta4,\Delta8}$ with fatty acid chain h20:0. The theoretical molecular formulas for these three structures were $C_{40}H_{76}NO_9$ (molecular weight 714), $C_{40}H_{78}NO_9$ (molecular weight 716) and $C_{44}H_{86}NO_9$ (molecular weight 772). Comparing the data from our study with the established data, the glucosylceramide isolated from wheat gluten in our study had one major structure with sphingoid base $d18:2^{\Delta4,\Delta8}$ and fatty acid chain h20:0 (molecular formula $C_{44}H_{86}NO_9$).

In this study, only one major peak at $[M+Na]^+$ m/z 793.84 (corresponding to the structure of $C_{44}H_{86}NO_9$) was found, and the peak at m/z 737.90 (corresponding to the structure of $C_{40}H_{78}NO_9$) was barely detectable. However, in the study conducted by Sullards et al. (2000), the peaks corresponding to these two structures had similar height. In the study performed by Sugawara and Miyazawa (1999), the peak of $C_{40}H_{78}NO_9$ was even higher than that of $C_{44}H_{86}NO_9$. Sugawara and Miyazawa (1999) suggested that the

concentration of glycolipids in plants could vary tremendously depending on the cultivar, growth condition, stage of development and harvested days. Therefore, the results from these three studies may imply the presence of different structures of glucosylceramide in wheat from different sources. Our result may represent the typical structure of glucosylceramide in commercial wheat gluten available in the American market.

From TLC and HPLC analyses, fractionation by silica gel column chromatography and saponification under mild alkaline conditions removed most of neutral lipids, phospholipids and some other glycolipids from total lipids so that sphingolipids were enriched by these procedures. The sphingolipid-enriched lipid fraction, which was one of an ingredient of Diet E in BBdp rats feeding study, contained enriched glucosylceramide.

BBdp Rats Feeding Study

In all NTP-2000, WG and WGSLF groups, there was one rat died because of some unknown reasons before they developed diabetes. Therefore, the final rat numbers on HC, NTP-2000, WG, WGSLF and HC+SL diets were 20, 19, 19, 19 and 20, respectively.

The body weight of rats was recorded on weekly basis from one week after the rats were treated with different diets. Since the body weight of male rats was much greater than that of female rats, the males and females were considered separately. Body weight over time in male and female BBdp rats is shown in Figure 7 and 8 respectively. Since the rats on WGSLF diet had lower body weight from the beginning, i.e. one week after diet treatment, the gain of body weight compared to the initial weight at different stages was calculated and compared. The results of body weight gain in male and female rats are shown in Table 3 and 4. The male rats on WGSLF had a significant lower rate of body weight gain within the first five weeks of feeding study, but after five weeks, there was no significant difference on weight gain rate among the male rats in these five groups. The rate of body weight gain of female rats had a similar pattern. There were some differences within the first five weeks, but no significant difference on weight gain rate was found after five weeks of diet treatment among all the female rats in these five groups.

According to the data in Table 3 and 4, rats on WGSLF diet gained weight more slowly compared to those on most of the other diets within the first five weeks of diet

treatment. The rats on WGSLF diet were found to refuse to eat or eat very little at the beginning of the feeding study. This seemed to be caused by the poor taste of wheat gluten after lipid extraction with chloroform-methanol mixture. Actions were taken by coating the pellets with a sugar-based, artificial maple syrup-flavored liquid to make this diet more palatable. It seemed that the rats in this group got used to this diet gradually, and the rate of their weight gain caught up with that of the rats in other groups even though their body weight was still lower. Diabetogenesis has been shown to be an accumulative process which involves the whole period from the beginning of puberty to late adolescence (Scott et al., 1997). Therefore, even the rats on WGSLF did not have sufficient food intake at the beginning, this would not affect the outcome of the feeding study since these rats started to eat normally after the actions were taken to make the diet more palatable.

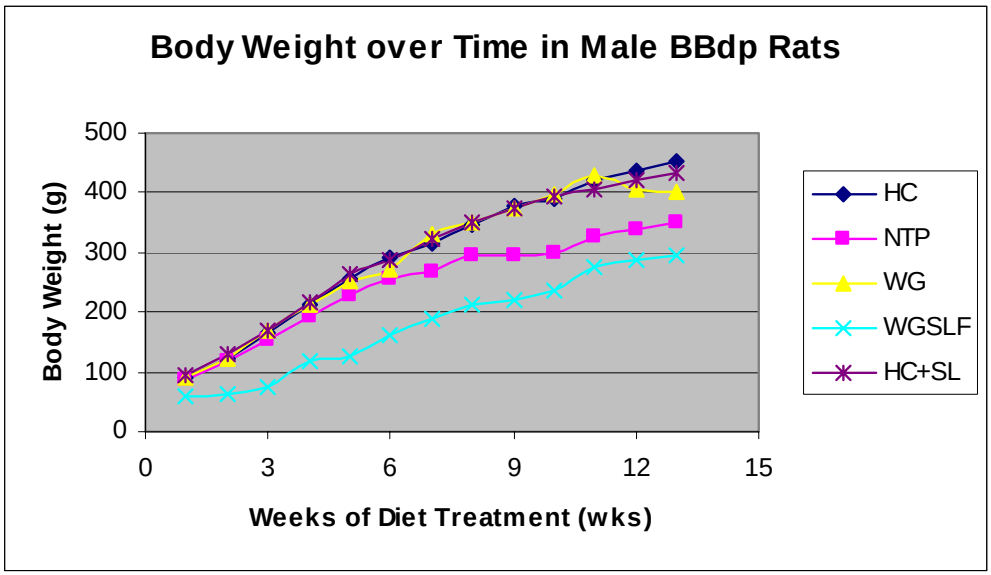


Figure 7. Body weight over time in male BBdp rats

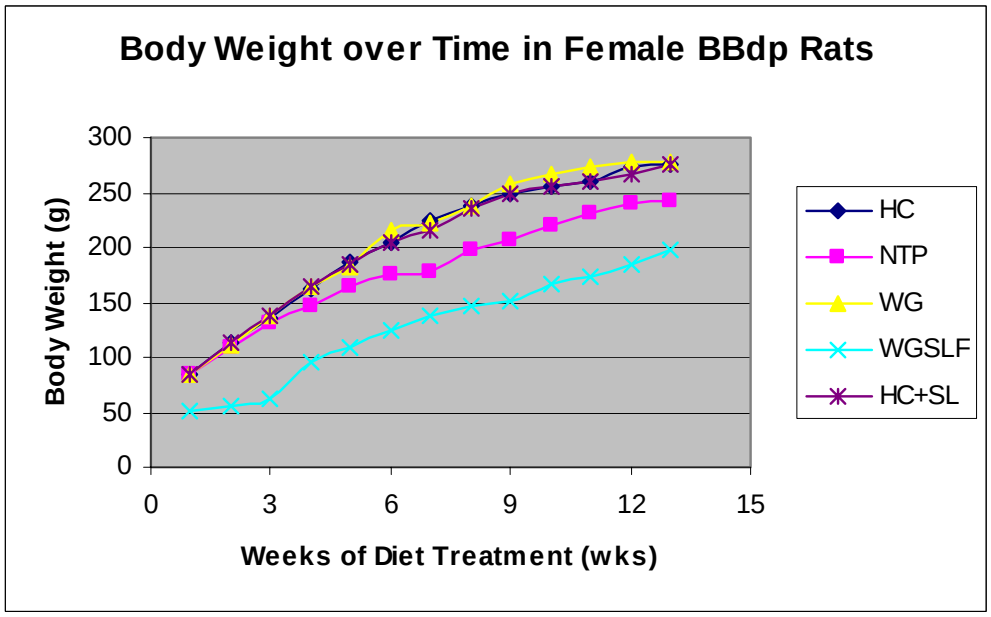


Figure 8. Body weight over time in female BBdp rats

Table 3. Effect of diet on the body weight gain of male BBdp rats

Diet	Gain of body weight (g)				
	Wk 2	Wk 3	Wk 4	Wk 5	Wk 6-13
HC (n=10)	33.6	76.1 [a]	122.0 [a]	167.1 [a]	
NTP-2000 (n=10)	31.8	67.9 [a]	103.9 [a]	140.2 [a]	No
WG (n=10)	34.7	77.6 [a]	121.6 [a]	159.1 [a]	Significant
WGSLF (n=11)	6.6	15.9 [b]	59.0 [b]	67.5 [b]	Differences
HC+SL (n=10)	34.6	76.6 [a]	121.5 [a]	168.0 [a]	

All values were reported as mean and were analyzed by two-way ANOVA. Comparison was made between five groups only at the same stage of the feeding study (within the same column as shown in the table above). There were significant differences when groups were marked with different letters a or b ($P<0.05$).

Table 4. Effect of diet on the body weight gain of female BBdp rats

Diet	Gain of body weight (g)				
	Wk 2	Wk 3	Wk 4	Wk 5	Wk 6-13
HC(n=10)	28.5	52.3 [a]	77	102.2 [a]	
NTP-2000(n=10)	24.3	46.5 [ab]	63.1	79.9 [ab]	No
WG(n=10)	26.5	52.8 [a]	78.5	98.1 [a]	Significant
WGSLF(n=9)	4.4	12.3 [b]	45.7	57.3[b]	Difference
HC+SL(n=10)	28.4	53.8 [a]	79.4	100.8[a]	

All values were reported as mean and were analyzed by two-way ANOVA. Comparison was made between five groups only at the same stage of the feeding study (within the same column as shown in the table above). There were significant differences when groups were marked with different letters a or b ($P<0.05$).

Table 5. Pancreas weight of non-diabetic female BBdp rats on different diets

Groups	HC (n=8)	NTP-2000 (n=3)	WG (n=6)	WGSLF (n=4)	HC+SL (n=5)
Pancreas Wt (mg)	677.3 ± 44.2	789.2 ± 141.1	770.1 ± 56.44	645.9 ± 40.9	728.6 ± 75.0

All values were reported as mean ± SEM and were analyzed by one-way ANOVA. n was the number of non-diabetic female rats at the end of the study. P>0.05.

Table 6. Onset age of diabetes among BBdp rats on different diets

Diet	HC (n=6)	NTP-2000 (n=11)	WG (n=11)	WGSLF (n=11)	HC+SL (n=8)
Diabetes Onset Age (days)	88 ± 6	82 ± 3	78 ± 6	79 ± 3	77 ± 5

All values were reported as mean±SEM and were analyzed by one-way ANOVA. n was the number of diabetic rats at the end of study. P>0.05.

Only one and two male rats survived without developing diabetes at the end of feeding study in NTP-2000 group and WG group respectively. These numbers are not great enough for statistic analysis. Therefore, the pancreas weight of non-diabetic rats at the end of study was only compared within female rats in different diet groups. The results are shown in Table 5. There was no significant difference between any two groups among these five diet groups. This suggested that different diets had no effect on the weight of pancreas in BBdp rats.

The onset age of diabetes in BBdp rats in the five diet groups is shown in Table 6. Rats on WG diet and WGSLF diet had almost the same diabetes onset age. Though there were variations among these five groups, especially the addition of wheat sphingolipids into hydrolyzed casein diet caused the onset age of diabetes eleven days earlier compared to hydrolyzed casein diet only, there were no statistically significant differences between any two groups among all these five diet groups ($P > 0.05$).

Survival curve of rats in different groups is shown in Figure 9. Rats on HC diet had a significantly higher survival rate compared to those on NTP-2000 diet. No significant difference was found between WG and WGSLF or HC and HC+SL groups, suggesting that the removal of sphingolipids from the diet or the addition of sphingolipids into the diet had no effect on the survival rate in BBdp rats.

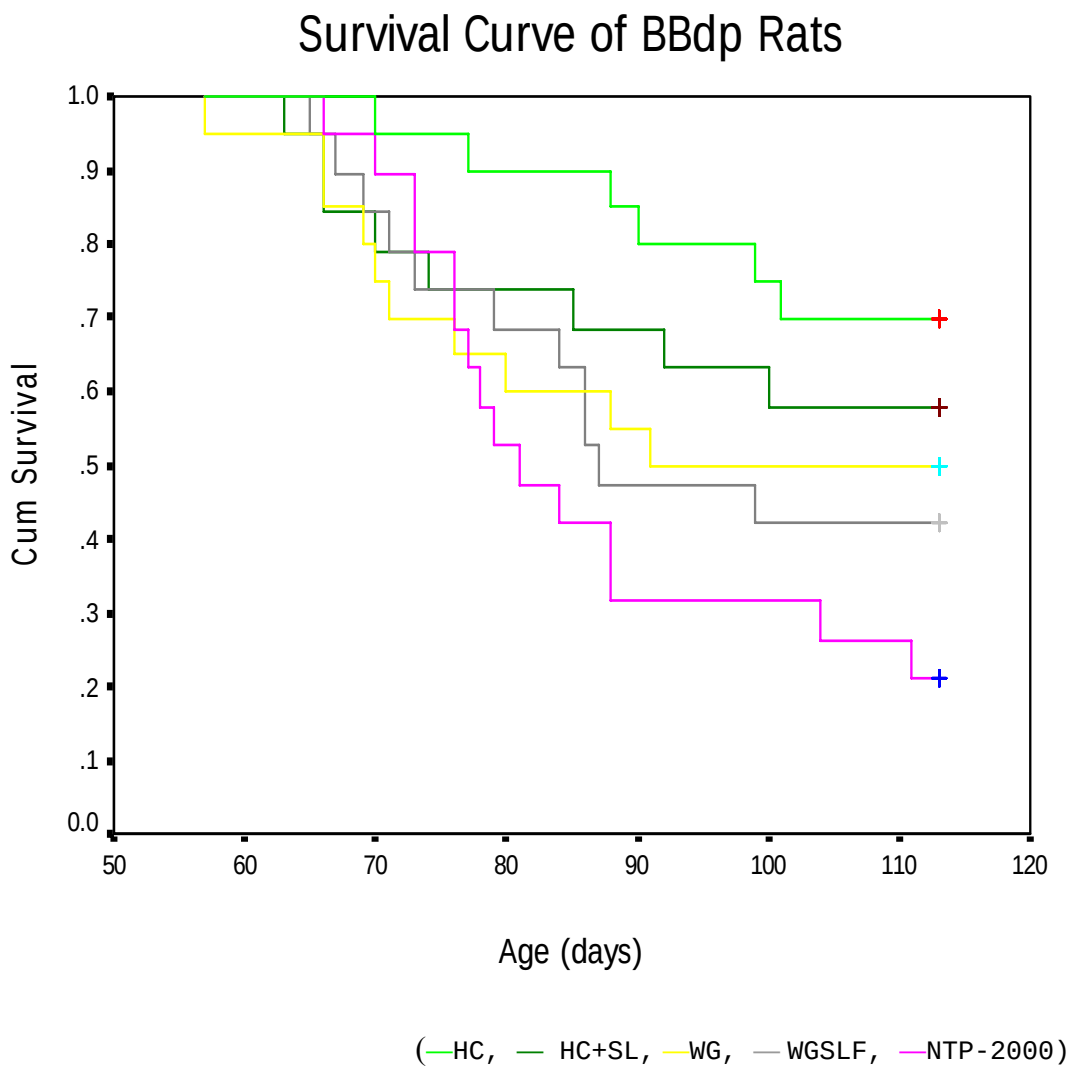


Figure 9. Survival curve of BBdp rats on different diets

Diabetes incidence of BBdp rats in different groups at different stages is shown in Table 7. Among these five diet groups, the rats on NTP-2000 diet had the highest diabetes incidence from 80 days to 125 days. Rats on WG diet and WGSLF diet had significantly higher diabetic incidence than those on HC diet at all the stages. There was no significant difference between WG and WGSLF diets at any stages. Interestingly, HC+SL diet caused a significantly higher diabetes incidence compared to HC diet at 70 days, 80 days and 100 days, while there was no statistically significant difference between these two groups at 90 days and after 100 days, though the rats on HC+SL diet still had a higher diabetes incidence than those on HC diet (30% vs 20% at 90 days, and 40% vs 30% after 100 days).

Table 7. Incidence of diabetes in BBdp rats at different stages of the feeding study

Age	No.	HC (n=20)	NTP-2000 (n=19)	WG (n=19)	WGSLF (n=19)	HC+SL (n=20)
70 day	N	1	2	5	3	4
	%	5.0	10.5	26.3	15.8	20.0
80 day	N	2	9	8	6	5
	%	10.0	47.4	42.1	31.6	25.0
90 day	N	4	13	9	10	6
	%	20.0	68.4	47.4	52.6	30.0
100 day	N	5	13	10	11	8
	%	25.0	68.4	52.6	57.9	40.0
110 day	N	6	14	10	11	8
	%	30.0	73.7	52.6	57.9	40.0
125 day	N	6	15	11	11	8
	%	30.0	78.9	57.9	57.9	40.0

(N=number of diabetic rats, %=the percentage of diabetic rats)

In this study, two control diets were used. HC diet was a negative control diet, since all the previous studies showed that this diet had a preventive effect on the development of type I diabetes in BBdp rats. NTP-2000 diet, which contained 37.3% of wheat product and no milk proteins, was used as a positive control, because previous studies showed this diet caused a very high diabetes incidence in BBdp rats (Scott, 1996; Beales et al., 2002). These findings were also confirmed by the current study. The rats on HC diet had the lowest diabetes incidence, which was only 30% compared to 79% in the rats on NTP-2000 diet at 125 days of age. WG diet, which contains 23.4% of wheat gluten, caused a diabetic incidence (58%) lower than the NTP-2000 diet but higher than HC diet. This result further confirmed that wheat products had a diabetes-triggering effect on BBdp rats, and this effect was concentration-related.

We failed to see any significant differences in the onset age of diabetes among BBdp rats in the five diet groups. Rats in the HC group had the latest diabetes onset age (88 days), which was ten days earlier than that in WG group. The addition of wheat sphingolipids fraction to the HC diet brought the mean onset age of diabetes eleven days earlier compared to the HC diet. However, these differences were not statistically significant. From previous studies (Scott, 1996), HC diet caused a significant delay on the diabetes onset age compared to NIH diet, which contains about 33% of wheat products (106 vs 80 days). However, we did not see this in the current study. The reason for this difference is not clear. It could be partially explained by the different time length of studies. The time length of the current study was 125 days, while the previous studies (Scott, 1996) continued until rats were 130-160 days of age.

The survival curve demonstrated that the overall survival rate of BBdp rats on HC diet was significantly higher than that on NTP-2000 diet. However, the removal of lipid from wheat gluten or the addition of wheat sphingolipids fraction into the diet did not change the overall survival rate. It also did not change the ultimate diabetes incidence in BBdp rats, as shown in Table 7 (at 125 days). However, the addition of wheat sphingolipid-enriched fraction to the HC diet significantly increased diabetes incidence compared to the HC diet at 70 days, 80 days and 100 days of age in BBdp rats. This suggested that wheat sphingolipids might increase diabetes incidence in BBdp rats at early

stages (i.e. before 80 days of age), but not at later stages of the feeding study (i.e. after 100 days of age) and it did not change the ultimate diabetes incidence. The promoting effect was not very solid at middle stages, since at 90 days, there was no significant difference between HC+SL group and HC group on diabetes incidence, though the incidence of diabetes was still higher in HC+SL group.

In a study conducted by Coleman et al. (1990), the investigators found that the incorporation of a chloroform-methanol extract from OG96 diet (contained 38.9% of wheat) into a HC diet significantly increased the ultimate diabetes incidence in NOD mice. It is possible that some other lipid fractions which were removed by column chromatography and saponification may also contribute to the development of type I diabetes. Most likely, wheat sphingolipids may promote, but not trigger, type I diabetes in BBdp rats. It might interact with some diabetes triggers in the diet, and bring their effect quicker so that this disease could be observed at an earlier stage in these animals, but it does not determine the ultimate diabetes incidence. There must be something else unknown in the wheat gluten that is responsible for the development of type I diabetes. This also suggests that type I diabetes is caused by multiple factors.

No significant difference was found between the rats on WG diet and WGSLF diet at any stages with regard to diabetes incidence. A possible explanation for the unchanged diabetes incidence in WGSLF group compared to WG group was that there were still some diabetes-causing substances in wheat gluten after chloroform-methanol extraction. These substances might not dissolve in chloroform-methanol mixture so they were not extracted by this mixture. Wheat proteins/peptides seem to be very possible candidates for these substances. Proline is one of the most abundant amino acid in wheat proteins (Sugiyama et al., 1985), which makes wheat proteins good substrates for dipeptidyl peptidase IV (DPP IV) (Hausch et al., 2002). DPP IV is a protease widely distributed in mammalian tissues such as small intestine and kidney (Lambeir et al., 2001). This enzyme selectively removes the N-terminal dipeptide from polypeptides with proline in the second position (Faust et al., 2003). DPP IV can be spontaneously released to act to the extracellular environment (Pereira et al., 2003). Therefore, it is conceivable that the human or animals with higher consumption of wheat products may have a higher level of activity of DPP IV in their guts.

One of this enzyme's substrates is glucagon-like peptide-1 (GLP-1), a peptide hormone released from the intestinal mucosa (Deacon et al., 1995). GLP-1 has an important effect on β-cells by stimulating the proliferation of β-cells, enhancing the differentiation of new β-cells and inhibiting β cell apoptosis (Holst, 2003). Recent studies showed that treating BBdp rats with DPP IV inhibitors resulted in a delay of onset of diabetes and improvement of glucose tolerance in these animals (Pospisilik et al., 2003). The GLP-1 content in intestinal tract in BBdp rats was found to be lower than that in the control rats (Malaisse et al., 2002), which might result from a proinflammatory state of the gastrointestinal tract that preceded the pancreatic insulitis (Cancelas et al., 2002).

In the current study, we noticed that some rats had inflamed or rubbery gut. The normal gut is soft and pliable to the touch, while rubbery gut is thickened, opaque, reddish and always accompanied with a leaky gut (personal communication between W.E. Barbeau and F.W. Scott). There were more rats with inflamed or rubbery gut on wheat containing diets than on HC or HC+SL diets, with six rats on NTP-2000 diet, eight rats on WG diet, seven rats on WGSLF diet, and four rats on both HC and HC+SL diets. The gut tissues from these rats were collected for our follow-up studies which would analyze the DPP IV activity and GLP-1 content.

This study is our first attempt to determine which substances in wheat cause type I diabetes in BBdp rats. In this study, we only examined if a wheat sphingolipid-enriched lipid fraction could affect the diabetes incidence and initial age of onset of diabetes in BBdp rats. We found this sphingolipid fraction could promote type I diabetes at early stages of BBdp rat feeding study but it did not change the ultimate diabetes incidence. In addition, the removal of lipids from wheat gluten did not cause a decrease in diabetes incidence in these rats. Most likely, the interaction between sphingolipids and some other unknown substances in wheat gluten are responsible for the development of type I diabetes in BBdp rats. Unfortunately, at this point we do not have the chance to do some tissue analysis to determine the degree of β cell insulitis and to examine the intestinal tract for the GLP-1 content and DPP IV activity. The pancreases and gut samples were saved for our follow-up studies.

Summary

The findings from this study are as follows:

1. It was confirmed by this study that wheat-containing diet caused higher diabetes incidence in BBdp rats compared to casein based diet. BBdp rats on NTP-2000 diet had the highest diabetes incidence, while rats on HC diet had the lowest diabetes incidence. WG diet, which contains less wheat gluten compared to NTP-2000 diet, caused an incidence of diabetes less than NTP-2000 diet but higher than HC diet;
2. There was no significant difference with regard to the onset age of diabetes among BBdp rats in the five diet groups;
3. The addition of wheat sphingolipid-enriched lipid fraction to the HC diet significantly increased diabetes incidence in BBdp rats at the early stages of the feeding study compared to the HC diet only. However, the ultimate diabetes incidence was not changed by sphingolipids addition. Wheat sphingolipids seemed to act as a possible promoter rather than a trigger for type I diabetes in BBdp rats;
4. The removal of the lipid fraction from wheat gluten did not change the incidence of diabetes compared to wheat gluten based diet at any stages of the feeding study. There were still some unknown diabetes-causing substances remaining in wheat gluten after chloroform-methanol extraction.

Limitations

Due to the low content of sphingolipids in wheat gluten, only a sphingolipid-enriched lipid fraction from wheat gluten was extracted and used in BBdp rats feeding study. This fraction was not a pure form of sphingolipids. Therefore, the contribution of other components in that fraction to the onset of type I diabetes was not taken into consideration.

Suggestions for Future Research

In this study, we only used a sphingolipid-enriched lipid mixture but not a pure form of sphingolipids BBdp rats feeding study. In addition, we did not do the tissue analysis. These problems would be solved by our follow-up studies:

1. Analysis of pancreatic tissues for the severity of β cell insulitis;
2. Analysis of gut tissues for GLP-1 content and DPP IV enzymatic activity;
3. Cell culture studies using pure wheat sphingolipids, especially glucosylceramides, to examine their effect on β-cell growth, β-cell apoptosis and insulin secretion; and
4. Identification of wheat proteins/peptides that affect the DPP IV activity and the destruction of pancreatic β cells.

Chapter 6: References

Akerblom HK, Knip M, 1998, Putative environmental factors in type I diabetes, Diabetes/Metabolism Reviews, 14:31-67

Almawi WY, Tamim H and Azar ST, 1999, T helper type 1 and 2 cytokines mediate the onset and progression of type I (insulin-dependent) diabetes, J. Clin. Endocrinol. Metab., 84: 1497-1502

AOAC 1980, Official Methods of Analysis, 14th edition, Association of official analytical chemists, Alington, VA

Atkinson MA, Bowman MA, Kao KJ, Campbell L, Dush PJ, Shah SC, Simell O and Maclaren NK, 1993, Lack of immune responsiveness to bovine serum albumin in insulin-dependent diabetes, N Engl J Med, 329:1853-1858

Atkinson MA and Maclaren NK, 1990, What causes diabetes? Scientific American, 263(1): 62-63, 66-71

Barnett AH, Eff C, Leslie RDG, Pyke DA, 1981, Diabetes in identical twins: a study of 200 pairs, Diabeologia, 20:87-93

Beales PE, Elliott RB, Flohe S, Hill JP, Kolb H, Pozzilli P, Wang GS, Wasmuth H and Scott FW, 2002, A multi-centre, blinded international trial of the effect of A(1) and A(2) beta-casein variants on diabetes incidence in two rodent models of spontaneous Type I diabetes, Diabetologia, 45:1240-1246.

Beppu H, Winter WE, Atkinson MA, Maclaren NK, Fujita K and Takahashi H, 1987, Bovine albumin antibodies in NOD mice, Diabetes Research, 6:67-69

Berlin C, Berg EL, Briskin MJ, Andrew DP, Kilshaw PJ, Holzmann B, Weissman IL, Hamann A and Butcher E, 1993, α4β7 integrin mediates lymphocyte binding to the mucosal vascular addressin MAdCAM-1, Cell, 74:185-195

Beyan H, Buckley LR, Yousaf N, Londei M and Leslie RD, 2003, A role for innate immunity in type 1 diabetes, Diabetes Metab. Res. Rev., 19:89-100

Bodansky HJ, Staines A, Stephenson C, Haigh D, Carwright R, 1992, Evidence for an environmental effect in the aetiology of insulin dependent diabetes in a transmigratory population, BMJ, 304:1020-2

Buesa-Gomez J, Torre J, Dyrberg T, Landin-Olsson M, Mauseth RS, Lernmark A, and Oldstone MBA, 1994, Failure to detect genomic viral sequences in pancreatic tissues from two children with acute-onset diabetes mellitus, J. of Medical Virology, 42:193-197

Cancelas J, Sancho V, Villanueva-Penacarrillo M, Courtois P, Scott FW, Valverde I and Malaisse WJ, 2002, Glucaon-like peptide 1 content of intestinal tract in adult rats injected with streptozotocin either during neonatal period or 7 d before sacrifice, Endocrine, 19:279-286

Chen H, Born E, Mathur SN, Johlin FC Jr and Field FJ, 1992, Sphingomyelin content of intestinal cell membranes regulates cholesterol absorption. Evidence for pancreatic and intestinal cell sphingomyelinase activity, Biochem. J., 286:771-777

Christie WW, 1996, Plant glycolipids: structure, isolation and analysis, in Advances in lipids methodology – three, Chap.6, P 247-277 , The Oily Press Ltd, Dundee, Scotland

Christie WW, 1992, Fatty acids and lipids: structures, extraction and fractionation into classes, in Gas chromatography and lipids: a practical guide, Chap. 2, P 25-27, The Oily Press Ltd, Ayr, Scotland

Christie WW, 1985, Rapid separation and quantification of lipid classes by HPLC and mass (light-scattering) detection, J. Lipid Res., 26:507-512

Christie WW, 1973, The structure, chemistry and occurrence of lipids in Lipid analysis: isolation, separation, identification and structural analysis of lipids, Chap. 1, P 17-29, Pergamon press Ltd, Oxford, England

Coleman DL, Kuzava JE and Leiter EH, 1990, Effect of diet on incidence of diabetes in nonobese diabetic mice, Diabetes, 39:432-436

Deacon CF, Johnsen AH, Holst JJ, 1995, Degradation of glucagon-like peptide-1 by human plasma in vitro yields an N-terminally truncated peptide that is a major endogenous metabolite in vivo, J Clin Endocrinol Metab., 80(3):952-957

Delovitch TL and Singh B, 1997, The nonobese diabetic mouse as a model of autoimmune diabetes: immune dysregulation gets the NOD, Immunity, 7: 727-738

Dressler KA, Mathias S and Kolesnick RN, 1992, Tumor necrosis factor-α activates the sphingomyelin signal transduction pathway in a cell-free system, Science, 255:1715-1718

Duan R, Cheng Y, Yang L, Ohlsson L and Nilsson, 2001, Evidence for specific ceramidase present in the intestinal contents of rats and humans, Lipids, 36:807-812

Duan R, Nyberg L and Nilsson, 1995, Alkaline sphingomyelinase activity in rat gastrointestinal tract: distribution and characteristics, Biochim Biophys Acta, 1259:49-55

Eisenbarth GS, and Lafferty KJ, 1996, Human type I diabetes mellitus: genetic susceptibility and resistance, Chap 7, P134-152, In Type I diabetes: molecular, cellular, and clinical immunology, Oxford University Press, Inc., New York

Eizirik DL and Darville MI, 2001, β-cell apoptosis and defense mechanisms: lessons from type I diabetes, Diabetes, 50 (suppl. 1):S64-S69

Elgert KD, 1996, Autoimmunity, In Immunology: understanding the immune system, Chap. 16, P 315-320, Wiley-Liss, Inc., New York, NY

Elliott RB and Martin JM, 1984, Dietary protein: a trigger of insulin-dependent diabetes in the BB rat, Diabetologia, 26:297-299

Elliott RB, Reddy SN, Bibby NJ and Kida K, 1988, Dietary prevention of diabetes in the non-obese diabetic mouse, Diabetologia, 31:62-64

Faust J, Fuchs P, Wrenger S, Reinhold D, Stockel-Maschek A, Kahne T, Ansorge S, Neubert K, 2003, Dipeptidyl peptidase IV inhibitors with the N-terminal MXP sequence: structure-activity-relationships, Adv. Exp. Med. Biol., 524:175-179

Fishbein JD, Dobrowsky RT, Bielawska A, Garrett S and Hannun YA, 1993, Ceramide-mediated growth inhibition and CAPP are conserved in Saccharomyces cerevisiae, J. Biol. Chem., 268:9255-9261

Foy CA, Quirke P, Williams DJ, Lewis FA, Grant PJ, Eglin R, and Bodansky HJ, 1994, A search for candidate viruses in type I diabetic pancreas using the polymerase chain reaction, Diabetic Medicine, 11:564-569

Feltbower RG, Bodansky HJ, McKinney PA, Houghton J, Stephenson CR, and Haigh D, 2002, Trends in the incidence of childhood diabetes in south Asians and other children in Bradford, UK, Diabetic Medicine, 19(2):162-166

Flohe SB, Wasmuth HE, Kerad JB, Beales PE, Pozzilli P, Elliott RB, Hill JP, Scott FW, Kolb H, 2003, A wheat-based, diabetes-promoting diet induces a Th1-type cytokine bias in the gut of NOD mice, Cytokine, 21(3):149-154

Folch J, Lees M and Stanley GHS, 1957, A simple method for the isolation and purification of total lipids from animal tissues, J. Biol. Chem., 226:497-509

Fujino Y and Ohnishi M, 1983, Sphingolipids in wheat grain, J. Cereal Sci., 1:159-168

Fujino Y and Ohnishi M, 1976, Constituents of ceramide and ceramide monohexoside in rice bran, Chem. Phys. lipids, 17:275-289

Funda DP, Kaas A, Bock T, Tlaskalova-Hogenova H and Buschard K, 1999, Gluten-free diet prevents diabetes in NOD mice, Diabetes/Metablism Research and Reviews, 15:323-327

Gale EAM, 2002, A missing link in the hygiene hypothesis? Diabetologia, 45:588-594

Goebel C, Kirchhoff K, Wasmuth H, Flohe S, Elliott RB, Kolb H, 1999, The gut cytokine balance as a target of lead toxicity, Life Sci., 64(24):2207-2214

Haimovitz-Friedman A, Kan C, Ehleiter D, Persaud RS, McLoughlin M, Fuks Z and Kolesnick RN, 1994, Ionizing radiation acts on cellular membranes to generate ceramide and initiate apoptosis, J. Exp. Med., 180:525-535

Hanninen A and Harrison LC, 2000, γδ T cells as mediators of mucosal tolerance: the autoimmune diabetes model, Immunol. Rev., 173:109-119

Hanninen A, Jaakkola I and Jalkanen S, 1998, Mucosal addressin is required for the development of diabetes in nonobese diabetic mice, J. Immunol., 160:6018-6025

Hanninen A, Salmi M, Simell O, Jalkanen S, 1996, Mucosa-associated (beta 7-integrinhigh) lymphocytes accumulate early in the pancreas of NOD mice and show aberrant recirculation behavior, Diabetes, 45(9):1173-1180

Hanninen A, Salmi M, Simell O, Jalkanen S, 1993, Endothelial cell-binding properties of lymphocytes infiltrated into human diabetic pancreas. Implications for pathogenesis of IDDM, Diabetes, 42(11):1656-1662

Hannun YA and Obeid LM, 1995, Ceramide: an intracellular signal for apoptosis, Trends Biochem. Sci., 20:73-77

Hardin JA, Donegan L, Woodman RC, Trevenen C and Gall DG, 2002, Mucosal inflammation in a genetic model of spontaneous type I diabetes mellitus, Can. J. Physiol. Pharmacol., 80:1064-1070

Harrison LC and Honeyman MC, 1999, Cow's milk and type 1 diabetes: the real debate is about mucosal immune function, Diabetes, 48:1501-1507

Hattori M, Buse JB, Jackson RA, Glimcher L, Dorf ME, Minami M, Makino S, Moriwaki K, Korff M, Kuzuya H, Imura H, Seidman JG, and Eisenbarth GS, 1986, The NOD mouse: recessive diabetogenic gene within the major histocompatibility complex, Science, 231:733-735

Hausch F, Shan, L, Santiago NA, Gray GM and Khosla C, 2002, Intestinal digestive resistance of immunodominant gliadin peptides, Am. J. Physiol. Gastrointest. Liver Physiol., 283: G996-G1003

Holst JJ, 2003, Implementation of GLP-1 based therapy of type 2 diabetes mellitus using DPP-IV inhibitors, Adv. Exp. Med. Biol.,524:263-79

Hoorfar J, Buschard K and Dagnaes-Hansen F, 1993, Prophylactic nutritional modification of the incidence of diabetes in autoimmune non-obese diabetic (NOD) mice, British J. Nurt., 69: 597-607

Jarvis WD, Kolesnick RN, Fornari FA, Traylor RS, Gewirtz DA and Grant S, 1994, Induction of apoptotic DNA damage and cell death by activation of the sphingomyelin pathway, Proc. Natl. Acad. Sci. USA, 91:73-77

Jung JH, Lee C, Kim YC and Kang SS, 1996, New bioactive cerebrosides from Arisaema amurense, J. Nat. Prod., 59:319-322

Kaneto H, Fum J, Seo HG, Suzuki K, Matsuoka T, Nakamura M, Tatsumi Y, Kamada T and Taniguchi N, 1995, Apoptotic cell death triggered by nitric oxide in pancreatic beta-cells, Diabetes, 44:733-738

Karjalainen J, Martin JM, Knip M, Ilonen J, Robinson BH, Savilahti E, Akerblom HK and Dosch H, 1992, A bovine albumin peptide as a possible trigger of insulin-dependent diabetes mellitus, N. Engl. J. Med., 327: 302-307

Kawano T, Cui J, Koezuka Y, Toura I, Kaneko Y, Motoki K, Ueno H, Nakagawa R, Sato H, Kondo E, Koseki H and Taniguchi, 1997, CD1d-restricted and TCR-mediated activation of Vα14 NKT cells by glycosylceramides, Science, 278:1626-1629

Kies C, Fox HM, Mattern PJ, Johnson VA and Schmidt JW, 1978, Comparative protein quality as measured by human and small animal bioassays of three lines of winter wheat, In Nutritional improvement of food and feed proteins, P 91-102, Friedman M Ed., Plenum Press, Inc., New York, NY

Kim SY, Choi YH, Huh H, Kim J, Kim YC and Lee HS, 1997, New antihepatotoxic cerebroside from Lycium chinenese fruits, J. Nat. prod., 60:274-276

Kolb H and Pozzilli P, 1999, Cow's milk and type I diabetes: the gut immune system deserves attention, Immunolgy Today, 20: 108-110

Kolesnick RN, Haimovitz-Friedman A and Fuks Z, 1994, The sphingomyelin signal transduction pathway mediates apoptosis for tumor necrosis factor, Fas, and ionizing radiation, Biochem. Cell Biol., 72:471-474

Kowluru A and Metz SA, 1997, Ceramide-activated protein phosphatase-2A activity in insulin-secreting cells, FEBS Lett., 41:179-182

Kraine MR and Tisch RM, 1999, The role of environmental factors in insulin-dependent diabetes mellitus: an unresolved issue, Environmental Health Perspectives, 107 (Suppl. 5): 777-781

Kuitunen M, Saukkonen T, Ilonen J, Akerblom HK and Savilahti E, 2002, Intestianl permeability to amannitol and lactulose in children with type I diabetes with the HLA-DQB11*02 allele, Autoimmunity, 35(5):365-368

Lambeir A, Durinx C, Proost P, Damme JV, Scharpe S and Meester ID, 2001, Kinetic study of the processing by dipeptidyl-peptidase IV/CD26 of neuropeptides involved in pancreatic insulin secretion, FEBS Letters, 507:327-330

Lee JY, Hannun YA and Obeid LM, 2000, Functional dichotomy of protein kinase C (PKC) in tumor necrosis factor-α signal transduction in L929 cells, J. Biol. Chem. 275: 29290-29298

Leiter EH, Serreze DV and Prochazka M, 1990, The genetics and epidemiology of diabetes in NOD mice, Immunology Today, 11: 147-149

Libman I, Songer T, LaPorte R, 1993, How many people in the U.S. have IDDM? Diabetes Care, 16:841-842

Lynch DV, 1993, Sphingolipids, in Lipid Metabolism in Plants, CRC Press, Florida, P 285-308

MacFarlane AJ, Burghardt KM, Kelly J, Simell T, Simell O, Altosaar I, Scott FW, 2003, A type 1 diabetes-related protein from wheat (Triticum aestivum). cDNA clone of a wheat storage globulin, Glb1, linked to islet damage, J. Biol. Chem., 278(1):54-63

Major CD, Gao Z and Wolf BA, 1999, Activation of the sphingomyelinase/ceramide signal transduction pathway in insulin-secreting β cells, Diabetes, 48: 1372-1380

Makino S, Kunimoto K, Muraoka Y, Mizushima Y, Katagiri K and Tochino Y, 1980, Breeding of a non-obese, diabetic strain of mice, Exp. Anim., 29(1):1-13

Malaisse WJ, Valverde I, Redondo A, Acitores A, Villanueva-Penacarrillo ML, Meuris S, Courtois P, Sener A, Scott FW, 2002, Glucagon-like peptide 1 content of the intestinal tract in BB rats, Diabetes, 51 (Suppl.) 2:2401

Mandrup-Poulsen T, 2001, β-cell apoptosis: stimuli and signaling, Diabetes, 50 (Suppl. 1):S58-S63

Marliss EB, Nakhooda AF, Poussier P and Sima AA, 1982, The diabetic syndrome of the 'BB' Wistar rat: possible relevance to type 1 (insulin-dependent) diabetes in man, Diabetologia, 22(4):225-232

Martin JM, Trink B, Daneman D, Dosch H-M and Robinson B, 1991, Milk proteins in the etiology of insulin-dependent diabetes mellitus (IDDM), Annals of Medicine, 23:447-452

Mayer EJ, Hamman RF, Gay EC, Lezotte DC, Savitz DA, and Klingensmith GJ, 1988, Reduced risk of IDDM among breast-fed children, Diabetes, 37:1625-1632

Mayes PA, 2000, Bioenergetics: The role of ATP, in "Harper's Biochemistry", 25th Edition, Chap 12, P 123-129, McGraw-Hill Companies

Merrill AH Jr, Schmelz EM, Wang E, Dillehay DL, Rice LG, Meredith F and Riley RT, 1997a, Importance of sphingolipids and inhibitors of sphingolipid metabolism as components of animal diets, J. Nutr., 127: 830S-833S

Merrill AH Jr, Schmelz EM, Dillehay DL, Spiegel S, Shayman JA, Schroeder JJ, Riley RT, Voss KA and Wang E, 1997b, Sphingolipids – the enigmatic lipid class: biochemistry, physiology, and pathophysiology, Toxicol. Appl. Pharmacol., 142:208-225

Naidenko OV, Koezuka Y and Kronenberg M, 2000, CD1-mediated antigen presentation of glycosphingolipids, Micro. Infec., 2:621-631

Nieda M, Nicol A, Koezuka Y, Kikuchi A, Takahashi T, Nakamura H, Furukawa H, Yabe T, Ishikawa Y, Tadokoro K and Juji T, 1999, Activation of human Vα24NKT cells by α-glycosylceramide in a CD1d-restricted and Vα24TCR-mediated manner, Human Immunol., 60:10-19

Nishimura T, Kitamura H, Iwakabe K, Yahata T, Ohta A, Sato M, Takeda K, Okumura K, Kaer LV, Kawano T, Taniguchi M, Nakui M, Sekimoto M and Koda T, 2000, The interface between innate and acquired immunity: glycolipid antigen presentation by CD1d-expressing dendritic cells to NKT cells induces the differentiation of antigen-specific cytotoxic T lymphocytes, Int. Immunol., 12(7):987-994

Nilsson A, 1968, Metabolism of sphingomyelin in the intestinal tract of the rat, Biochim Biophys Acta, 164: 575-584

Nilsson A, 1969a, Metabolism of cerebrosides in the intestinal tract of the rat, Biochim Biophys Acta, 187: 113-121

Nilsson B, 1969b, The presence of sphingomyelin- and ceramide-cleaving enzymes in the small intestinal tract, Biochim Biophys Acta, 176:339-347

Nyberg L, Duan R, Axelson J and Nilsson A, 1996, Identification of an alkaline sphingomyelinase activity in human bile, Biochim Biophys Acta, 1300:42-48

Obeid LM, Linardic CM, Karolak LA and Hannun YA, 1993, Programmed cell death induced by ceramide, Science, 259: 1769-1771

Oikawa Y, Shimada A, Yamada S, Motohashi Y, Nakagawa Y, Irie J, Maruyama T and Saruta T, 2002, High frequency of $V\alpha24^{+}V\beta11^{+}$ T-cell observed in type 1 diabetes, Diabetes Care, 25:1818-1823

Okazaki T, Bielawska A, Bell RM and Hannun YA, 1990, Role of ceramide as a lipid mediator of 1α, 25-Dihydroxyvitamin D3-induced HL-60 cell differentiation, J. Biol. Chem. 265: 15823-15831

Onkamo P, Vaananen S, Karvonen M, Tuomilehto J, 1999, Worldwide increase in incidence of type I diabetes - the analysis of the data on published incidence trends, Diabetologia, 42:1395-1403

Parfrey NA, Prud'homme GJ, Colle E, Fuks A, Seemayer TA and Guttmann RD, 1989, Immunologic and genetic studies of diabetes in the BB rat, Crit. Rev. Immunol., 9(1):45-65

Pereira DA, Gomes L, El-Cheikh MC and Borojevic R, 2003, Dipeptidyl peptidase IV (CD26) activity in the hematopoietic system: differences between the membrane-anchored and the released enzyme activity, Braz. J. Med. Biol. Res., 36 (5), 567-578

Pettus BJ, Chalfant CE and Hannun YA, 2002, Ceramide in apoptosis: an overview and current perspectives, Biochim Biophys Acta, 1585:114-125

Pospisilik JA, Ehses JA, Doty T, McIntosh CH, Demuth HU, Pederson RA, 2003, Dipeptidyl peptidase IV inhibition in animal models of diabetes, Adv. Exp. Med. Biol., 524:281-291.

Pozzilli P, Signore A, Williams AJK and Beales PE, 1993, NOD mouse colonies around the world – recent facts and figures, Immunol. Today, 14:193-196

Rabinovitch A, Suzrez-Pinzon WL., Shi Y, Morgan AR and Bleackley RC, 1994, DNA fragmentation is an early event in cytokine-induced islet beta-cell destruction, Diabetologia, 37:733-738

Rao GN, 1996, New diet (NTP-2000) for rats in the National Toxicology Program toxicity and carcinogenicity studies, Fundam Appl Toxicol., 32(1):102-8.

Reijonen H, Ilonen J, Michelson B, Akerblom HK, 1990, HLA-DQ beta-chair restriction fragment length polymorphism as a risk marker in type I (insulin-dependent) diabetes mellitus: a Finnish family study, Diabetologia, 33:357-362

Rewers M, 1991, The changing face of the epidemiology of insulin-dependent diabetes mellitus (IDDM): Research designs and models of disease causation. Ann. Med., 23:419-426

Rewers M, Laporte RE, Walczak M, Dmochowski K and Bogaczynska E, 1987, Apparent epidemic of insulin-dependent diabetes mellitus in Midwestern Poland, Diabetes, 36:106-113

Robinson BH, Dosch HM, Martin JM, Akerblom HK, Savilahti E, Knip M and Ilonen J, 1993, Diabetologia, 36: 364-368

Robyt JF and White BJ, 1987, Methods for determining biological molecules, in Biochemical techniques: theory and practice, Chap 7, P 243-245, Waveland Press, Inc., Prospect Heights, IL

Sang S, Kikuzaki H, Lapsley K, Rosen RT, Nakatani N and Ho CT, 2002, Sphingolipid and other constituents from almond nuts (prunus amuygalus batsch), J. Agric. Food Chem. 50: 4709-4712

Savilahti E, Tuomilehto J, Saukkonen TT, Akerblom HK, Virtala ET, 1993, Increased levels of cow's milk and β-lactoglobulin antibodies in young children with newly diagnosed IDDM, Diabetes Care, 16: 984-989

Schmelz EM and Merrill AH JR, Ceramides and ceramide metabolites in cell regulation: evidence for dietary sphingolipids as inhibitors of colon carcinogenesis, Nutrition, 14 (1998) 717-719

Schmelz EM, Crall KJ, Larocque R, Dillehay DL and Merrill AH Jr, 1994, Uptake and metabolism of sphingolipids in isolated intestinal loops of mice, J. Nutr., 124:702-712

Scott FW, 1996, Food-induced autoimmune diabetes, Diabetes Metab Rev, 12: 341-359

Scott FW, Cloutier HE, Kleemann R, Woerz-Pagenstert U, Rowsell P, Modler HW and Kolb H, 1997, Potential mechanisms by which certain foods promote or inhibit the development of spontaneous diabetes in BB rats: dose, timing, early effect on islet area, and switch in infiltrate from Th1 to Th2 cells, Diabetes, 46:589-598

Scott FW, Mongeau R, Hatina G, Trick KD, and Wojcinski Z, 1985, Diet can prevent diabetes in the BB rat, Diabetes, 34: 1059-1062

Shimabukuro M, Zhou Y, Levi M and Unger RH, 1998, Fatty acid-induced β-cell apoptosis: a link between obesity and diabetes, Proc. Natl. Acad. Sci. USA, 95:2498-2502

Sjoholm A, 1995, Ceramide inhibits pancreatic β-cell insulin production and mitogenesis and mimics the actions of interleukin-1β, FEBS Lett., 367:283-286

Somnay-Wadgaonkar K, Nusrat A, Kim HS, Canchis WP, Balk SP, Colgan SP and Blumberg RS, 1998, Immunolocalization of CD1d in human intestinal epithelial cells and identification of a β2-microglobulin-associated form, Int. Immunol., 11(3):383-392

Sperling P and Heinz E, 2003, Plant sphingolipids: structural diversity, biosynthesis, first genes and functions, Biochim Biophys Acta, 1632:1-15

Sugawara T and Miyazawa T, 1999, Separation and determination of glycolipids from edible plant sources by high-performance liquid chromatography and evaporative light-scattering detection, Lipids, 34:1231-1237

Sugiyama T, Rafalski A, Peterson D, Soll D A, 1985, Wheat HMW glutenin subunit gene reveals a highly repeated structure, Nucleic Acids Res., 13(24):8729-37

Sullards MC, Lynch DV, Merrill Jr AH and Adams J, 2000, Structure determination of soybean and wheat glucosylceramides by tandem mass spectrometry, J. Mass Spectrom. 35:347-353

Tatham AS, Miflin BJ and Shewry PR, 1985, The beta-turn conformation in wheat gluten proteins: relationship to gluten elasticity, Cereal Chem., 62 (5):405-412

Thosmas HE and Kay TWH, 2000, Beta cell destruction in the development of autoimmune diabetes in the non-obese diabetic (NOD) mouse, Diabetes Metab. Res. Rev., 16:251-261

Tisch R and McDevitt H, 1996, Insulin-Dependent Diabetes Mellitus, Cell, 85:291-297

Vaarala O, 1999, Gut and the induction of immune tolerance in type 1 diabetes, Diabetes Metab. Res. Rev., 15:353-361

Vesper H, Schmelz E, Nikolova-Karakashian MN, Dillehay DL, Lynch DV and Merrill AH, 1999, Sphingolipids in food and the emerging importance of sphingolipids to nutrition, J. Nutr., 129:1239-1250

Wagenknecht LE, Roseman JM, and Herman WH, 1991, Increased incidence of insulin-dependent diabetes mellitus following an epidemic of Coxsackievirus B5, Am. J. Epidemiol., 133 (10) :1024-1031

Wal YV, Corazza N, Allez M, Mayer LF, Iijima H, Ryan M, Cornwall S, Kaiserlian D, Hershberg R, Koezuka Y, Colgan SP and Blumberg RS, 2003, Delineation of a CD1d-restricted antigen presentation pathway associated with human and mouse intestinal epithelial cells, Gastroenterology, 124:1420-1431

Wang G, Gruber H, Smyth P, Pulido O, Rosenberg L, Duguid W and Scott FW, 2000, Hydrolyzed casein diet protects BB rats from developing diabetes by promoting islet neogenesis, J. Autoimmunity, 15:407-416

Wasmuth HE and Kolb H, 2000, Cow's milk and immune-mediated diabetes, Proc. Nutr. Soc., 59: 573-579

Weiner HL, 1997, Oral tolerance for the treatment of autoimmune diseases, Annu. Rev. Med., 48:341-351

Whelan RD and Parker PJ, 1998, Loss of protein kinase C function induces an apoptotic response, Oncogene, 16:1939-1944

Wieser H, 1996, Relation between gliadin structure and coeliac toxicity, Acta Paediatr. Suppl., 412:3-9

Yamauchi R, Aizawa K, Inakuma T and Kato K, 2001, Analysis of molecular species of glycolipids in fruit paste of red bell pepper (Capsicum annuum L.) by high-performance liquid chromatography-mass spectrometry, J. Agric. Food Chem., 49:622-627

Yang XD, Michie SA, Mebius RE, Tisch R, Weissman I and McDevitt HO, 1996, The role of cell adhesion molecules in the development of IDDM: implications for pathogenesis and therapy, Diabetes, 45:705-710

Yang XD, Michie SA, Tisch R, Karin N, Steinman L and McDevitt HO, 1994, A predominant role of integrin α4 in the spontaneous development of autoimmune diabetes in nonobese diabetic mice, Proc. Natl. Acad. Sci. USA, 91:12604-12608

Yang XD, Sytwu HK, McDevitt HO and Michie SA, 1997, Involvement of beta 7 integrin and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) in the development of diabetes in obese diabetic mice, Diabetes, 46 (10):1542-1547

Appendices

Appendix A

Determination of Crude Protein (Method 2.057 AOAC, 1980)

Equipment Needed:

1. Kjeldahl tubes
2. Digestion heaters
3. Buchi apparatus

Reagent Needed:

1. Concentrated sulfuric acid (H_2SO_4) (93-98%, nitrogen free)
2. Catalyst: 1:50 w/w copper sulfate-sodium sulfate (copper sulfate was the catalyst and sodium sulfate was added to elevate the boiling point)
3. Sodium hydroxide (NaOH) solution (nitrogen free): 450g solid NaOH dissolved in 1L water (specific gravity=1.36 or more)
4. Methyl-red methylene blue indicator: mix 2 parts 0.2% alcoholic methyl red solution with 1 part of 0.2% alcoholic methylene blue solution
5. Standard H_2SO_4 (about 0.1N)
6. Boric acid-methylene blue receiver solution: add 360g boric acid (crystals) and 48ml methyl-red-methylene blue indicator to 18L of water

Procedure:

1. Weigh 0.5-1g sample into digestion tubes.
2. Add ½ scoop of catalyst and 15ml concentrated H_2SO_4. Digestion tubes were placed into digestion blocks. The heaters were turned on. Samples were digested until clear blue solutions were obtained. Then samples were digested for additional 10 minutes and cooled.
3. Samples were placed under an automatic Buchi steam distillation and titration system.

4. A blank was run periodically, using all reagents except samples, and values were corrected appropriately.
5. Protein was determined as follows:

$$\% \text{ Nitrogen} = (V_1\text{-}V_0)\ N \times 1.4007 \times 100 / W$$

$$\% \text{ Crude Protein} = (V_1\text{-}V_0)\ N \times 1.4007 \times 6.25 \times 100 / W$$

Where

V_1 = Volumes of the standard H_2SO_4 used to titrate samples

V_0 = Volumes of the standard H_2SO_4 used to titrate the blank

N = Normality of the acid

1.4007 = Milliequivalent weight of nitrogen x 100(%)

6.25 = Protein conversion factor for wheat products

W = Weight of samples (g)

Appendix B

Determination of Crude Fat (Method 7.056 AOAC, 1980)

The equipment used was Soxtec fat extractor.

1. Samples were dried overnight at 90 ^{o}C and stored in dessicator.
2. Cups plus glass beads were dried at 90 ^{o}C and weighed after they were cooled to room temperature in dessicator.
3. Weigh 0.5-3g of samples directly into thimble.
4. Thimbles were put in holder. In each cup, 50ml of petroleum ether was added.
5. The thimble was lowed into the petroleum ether and boiled for 30 minutes.
6. The thimble was boiled for 60 minutes with the extraction knobs in the rinsing position.
7. The solvent was collected for 15 minutes.
8. The cups were released from extraction unit and kept under the hood until all ether was gone.
9. Cups were dried in 90 ^{o}C oven for 15-30 minutes and weighed after they were cooled to room temperature in dessicator.
10. Crude fat was determined as follows:

$$\% \text{ Crude Fat} = (W_2 - W_1) \times 100 / Ws$$

Where

W_2 = Weight of cup + glass beads + fat collected

W_1 = Weight of cup + glass beads

Ws = Weight of sample

Appendix C

Determination of Moisture (Method 7.007 AOAC, 1980)

1. Aluminum dishes and covers were dried for 1 hour at 135 ^{o}C, cooled in dessicator and were then weighed prior to use.
2. Samples (2-3g) were weighed into the tarred moisture dishes.
3. Dishes were uncovered and placed on oven shelf.
4. Samples were heated for 2 hours after the oven temperature had reached 135 ^{o}C.
5. After 2 hours, samples were covered with lids, removed from the oven and transferred to a dessicator. Dishes were weighed after they were cooled to room temperature.
6. Replicate determinations had to be within 0.2%.
7. Moisture values were calculated as follows:

$$\% \text{ Moisture} = [W_1-(W_2-W_0)] \times 100 / W_1$$

Where

W_1 = Weight of samples before drying

W_2 = Weight of dishes + samples after drying

W_0 = Weight of dishes

Appendix D

Determination of Ash (Method 7.009 AOAC, 1980)

An electric muffle furnace was used. The procedure was as follows:

1. Samples (2g) were weighed into a pre-weighed porcelain crucible.
2. Samples were placed in the muffle furnace. The muffle furnace was brought to 600^{o}C by several automated steps. The samples were incinerated under this temperature for 2 hours.
3. The crucibles were transferred to a dessicator and weighed immediately when they were cooled to room temperature.
4. Ash values were calculated as follows:

$$\% \text{ Ash} = (W_2 - W_0) \times 100 / W_1$$

Where

W_1 = Weight of sample

W_0 = Weight of crucible

W_2 = Weight of crucible + sample residue after burning

Appendix E

The Formulas of Five Diets in BBdp Rats Feeding Study

Hydrolyzed Casein Diet –Group A

Ingredients	Percent (%)	Weight in Grams
Corn Starch	53.0	7,420.0
Sucrose	12.0	1,680.0
Casein Hydrolysate	18.0	2,520.0
Soy Oil	7.0	980.0
Cellulose-Type Fiber	5.0	700.0
AIN-93G Mineral Mix	3.5	490.0
AIN-93G Vitamin Mix	1.0	140.0
Choline Bitartrate	0.2	28.0
L-Cystine	0.3	42.0
Total	100.0	14,000.0

NTP-2000 Diet – Group B

Ingredients	Percent (%)
Ground Wheat	22.26
Ground Corn	22.18
Wheat Middlings	15.0
Soybean Meal (49% protein)	5.0
Fish Meal (60% protein)	4.0
Dried Brewer's Yeast	1.0
Alfalfa Meal (17% protein)	7.5
Oat Hulls	8.5
Purified Cellulose	5.5
Corn Oil (without preservatives)	3.0
Soy Oil (without preservatives)	3.0
Sodium Chloride	0.3
Calcium Phosphate, Diabasic (USP	0.4
Calcium Carbonate (USP)	0.9
Choline Chloride (0% choline)	0.26
Methionine	0.2
AIN-93G Vitamin Premix	0.5
AIN-93G Mineral Premix	0.5

Wheat Gluten Diet – Group C

Ingredients	Percent (%)	Weight in Grams
Wheat Gluten (ICN)	23.40	3,276.0
Corn Starch	49.00	6,860.0
Sucrose	12.00	1,680.0
Soy Oil	6.10	854.0
Cellulose-Type Fiber	3.92	548.8
AIN-93G Mineral Mix	3.50	490.0
AIN-93G Vitamin Mix	1.00	140.0
Choline Bitartrate	0.20	28.0
L-Cystine	0.30	42.0
L-Lysine	0.50	70.0
L-Threonine	0.08	11.2
Total	100.00	14,000.0

Note: No Tert-butylhydroquinone added, soy oil contains 200 ppm BHA, BHT.

Diet is made to contain 18% of protein.

(Proximate analysis: %crude fat: 0.9%; %crude protein: 76.8%; %moisture: 6.2%; %ash: 0.9; %carbohydrate: 15.1)

Wheat Gluten (Sphingolipids-free) Diet – Group D

Ingredients	Percent (%)	Weight in Grams
Wheat Gluten (ICN)	23.60	3,304.0
Corn Starch	48.10	6,734.0
Sucrose	12.00	1,680.0
Soy Oil	6.80	952.0
Cellulose-Type Fiber	3.92	548.8
AIN-93G Mineral Mix	3.50	490.0
AIN-93G Vitamin Mix	1.00	140.0
Choline Bitartrate	0.20	28.0
L-Cystine	0.30	42.0
L-Lysine	0.50	70.0
L-Threonine	0.08	11.2
Total	100.0	14,000.0

Note: No Tert-butylhydroquinone added, soy oil contains 200 ppm BHA, BHT.

Diet is made to contain 18% of protein.

(Proximate analysis: %crude fat: 0.2%; %crude protein: 76.3%; %moisture: 6.6%; %ash: 1.05; %carbohydrate: 15.8)

Special Note: To make the diet more palatable, pellets were coated with a sugar-based, artificial maple syrup-flavored liquid.

Recommended Mixing Time: 1 hour

Special Instructions: Store at 4^{o}C

Hydrolyzed Casein + Sphingolipids Diet –Group E

Ingredients	Percent (%)	Weight in Grams
Corn Starch	53.0	7,420.0
Sucrose	12.0	1,680.0
Casein Hydrolysate	18.0	2,520.0
Soy Oil	3.4	473.0
Soy Oil Containing Wheat Sphingolipids (extracted from wheat gluten)	3.6	507.0
Cellulose-Type Fiber	5.0	700.0
AIN-93G Mineral Mix	3.5	490.0
AIN-93G Vitamin Mix	1.0	140.0
Choline Bitartrate	0.2	28.0
L-Cystine	0.3	42.0
Total	100.0	14,000.0

Note: For the addition of sphingolipids containing soy oil, split the amount in half (946/2 = 473 ml soy oil) in the first two batches of diet.

Appendix F

NIH-07 Open Formula Rodent Diet

Ingredients	Percent (%)
Dried Skim Milk	5
Fish Meal	10
Soybean Meal	12
Alfalfa Meal	4
Corn Meal	3
Ground #2 Yellow Shelled Corn	23.5
Ground Hard Winter Wheat	23
Wheat Middlings	10
Dried Brewers Yeast	2
Dried Molasses	1.5
Soybean Oil	2.5
Salt Mix	3
Vitamin Mix	0.5

Appendix G

AIN-76A Purified Rodent Diet (Hydrolyzed Casein Based)

Ingredients	Percent (%)
Casein	20
DL-Methionine	0.3
Cornstarch	45
Dyetrose	20
Cellulose	5
Corn Oil	5
Salt Mix	3.5
Vitamin Mix	1
Choline Bitartrate	0.2

Vita

Wenjuan Shi was born in September, 1972 in China. She graduated from University of Science and Technology of China in 1996, with a Bachelor of Science degree in Chemistry. After that, she had worked in the chemistry lab in Wyeth Nutritional Co. Ltd. for five years. She joined Virginia Polytechnic Institute and State University in the fall semester of 2001. She will receive a Master of Science degree in the major of Human Nutrition and Foods. She plans to pursue a PhD study in molecular nutrition/nutritional biochemistry field in the future.