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IDENTIFYING AND CHARACTERIZING AGENTS IN SOY THAT HAVE A POTENTIAL ROLE IN DIABETES DEVELOPMENT

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Thesis submitted to the Department of Biochemistry in partial fulfillment of the requirements for the degree of Master of Science

University of Ottawa
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ABSTRACT

It is now clear that different soy proteins induce type I (insulin-dependent) diabetes in both models of type I diabetes, BB (Biobreeding) rat and NOD (non-obese diabetic) mice. To examine which component (s) of soy proteins are diabetogenic, studies were designed to look at diabetes-related soy protein fractions. In a study of diabetes-prone and control BB (BioBreeding) rats, sera from BBc (non-diabetes-prone), BBdp (diabetes-prone), BBd-U (diabetic untreated) and BBd-T (diabetic insulin-treated) rats at different risk of developing diabetes were tested against the proteins extracted from different soy protein sources. A diabetes-related protein designated S10 was identified and reactivity against two protective bands designated S6 and S13 was associated with less risk of developing diabetes. Two separate cross sectional and prospective (blood samples collected at 45 d, 70 d and 149 d or at diagnosis) studies were carried out. Western blotting results in both studies confirmed that high S10 reactivity in the pre-diabetic period was associated with diabetes. Overall Western blot analyses showed interestingly that two soy protein fractions, S6 and S13, were related to resistance and reactivity against these two fractions may be protective and the S10 soy protein fraction appears to be highly diabetes-related and associated with diabetes. In studies using human serum, two sets of blood samples from newly diagnosed diabetic children showed the same association for the S10 soy protein fraction. In studies of the mechanisms related to the soy-diabetes interaction, early oral dosing with soy meal antigens and pre-absorption of soy antibodies with rat insulinoma cells were examined. Results showed that early oral dosing with soybean meal delayed the onset of diabetes and protected some of the animals from developing the disease. Pre-absorption studies showed there was an overall reduction in reactivity to soy antigens using pre-absorbed sera. This indicated some cross reactivity between food antigens and RIN cell proteins. Overall, we conclude that soybean proteins contain a diabetogenic component which could be the S10 fraction, A1a, A1b or the A2 subunits of the acidic subunit of 11S glycinin. Also the pattern of reactivity against soy proteins (no or low S13, low S6 and high S10 reactivity) can be used as a marker in the pre-diabetic period for prediction of diabetes in BBdp rats.

DEDICATION

This thesis is dedicated to my husband for his excellent support and encouragement throughout this work, and to my lovely daughter for her understanding and patience.

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ABBREVIATIONS

AAP	American Academy of Pediatrics
BB rat	Bio-Breeding rat
BBc	Bio-Breeding control (non-diabetes-prone rat)
BBdp	Bio-Breeding diabetes-prone rat
BBd-U	Bio-Breeding diabetic rat, untreated
BBd-T	Bio-Breeding diabetic rat, insulin-treated
BLG	β-lactoglobulin
DAB	diaminobenzidine
ELISA	enzyme-linked immunosorbent assay
EDTA	ethylenediamine tetra acetic acid
GAD	glutamic acid decarboxylase
GABA	γ-aminobutyric acid
HLA	human leucocyte antigen
IDDM	insulin-dependent diabetes mellitus
kDa	kilo Dalton
MHC	major histocompatibility complex
M.W.	molecular weight
NEDH	New England Deaconess Hospital (rats); source of RIN cell tumor
NIH	NIH-O7, standard cereal-based rodent diet
NOD	non-obese diabetic mouse
PMSF	phenylmethylsulfonyl fluoride
Rf	resolution factor
RIN cell	rat insulinoma cell line (β cell tumor)
SPF	specific pathogen free
SPI	soy protein isolate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SDS	sodium dodecyl sulfate
TEMED	N,N, N′, N′ -Tetramethylenediamine

INTRODUCTION

IDDM is an Autoimmune Disease

Type I diabetes or insulin dependent diabetes mellitus (IDDM) is an organ specific autoimmune disease in which the insulin secreting β cells are destroyed by an inflammatory cell infiltrate affecting the islets of Langerhans (Bottazzo 1984; Foulis, Liddle, Farguharson 1986). The discovery of a mononuclear cell infiltrate in pancreatic islets of children with IDDM first suggested a role for autoimmunity in type I diabetes (Gepts 1965). The presence of T-cells in the infiltrate and the increased levels of activated T-cells in the circulation of new onset type I diabetic patients and in islet lesions (Jackson, Morris, Eisenbarth 1982) also supports a role for activated T- cells in the pathogenesis of the disease. CD8+ T-cells formed the majority of infiltrating lymphocytes in the pancreas of a child with new onset diabetes (Bottazzo, Dean et al. 1985). Thus, T-cells are essential for development of diabetes and are important effector cells in the pathogenesis. However, several other cells also participate in the attack on the β cells including macrophages, dendritic cells and possibly epithelial cells and the possibility that lymphocytes may recruit other cells as the final effectors for β cell destruction cannot be excluded (Castaño and Eisenbarth 1990).

Diabetes is extremely rare before 9 months of age, has a peak incidence between 5 and 15 years of age, and thereafter declines sharply with some cases occurring in adults up to 40 years of age (Castaño and Eisenbarth 1990). Metabolic changes involve both glucose metabolism and β cell function and can be detected several years before the clinical onset of IDDM. During the prediabetic period, a decline in insulin secretion can be detected up to 11 years before the clinical onset of IDDM (Srikanta, Ganda, Eisenbarth et al., 1983). Decreased first phase insulin release following intravenous glucose

injection, the presence of diabetes risk genes in the major histocompatibility complex (MHC) class II region and increased levels of certain autoantibodies have been used to predict who will develop diabetes (Palmer 1993).

Type I diabetic patients have autoantibodies to numerous antigens such as glutamic acid decarboxylase (GAD), a 38 kDa protein, insulin, proinsulin, carboxypeptidase H, amylin, heat shock proteins, glucose transporter, thymic hormones, thyroid, adrenal cortex, and others (Castaño and Eisenbarth 1990; Palmer 1993). The presence of these antibodies is useful in predicting the course of β cell destruction, but it is difficult to know if these antigens have a primary role in initiating the immune response, or whether immune reactivity towards them is a secondary event resulting from their release from lysed β-cells (Atkinson and Maclaren 1993).

Most of the β-cell autoantigens detected thus far were identified by demonstrating antibodies against them in sera of diabetic subjects. A particularly important antigen of molecular mass 64 kDa was detected by immunoprecipitation of islet cell lysates (Baekkeskov, Neilson, Marner, Bilde, Ludvigsson and Lernmark 1982), and was biochemically characterized as glutamic acid decarboxylase (GAD), a neural enzyme that controls the biosynthesis of γ-aminobutyric acid (GABA) (Baekkeskov, Aanstoot, Christgau, Reetz, Solimena, Cascalho, Folli, Ritcher-Oleson and Conilli 1990). Studies in non-obese diabetic (NOD) mice, an animal model of spontaneous insulin-dependent diabetes, showed an early T-cell response to the 65 kDa isoform of the enzyme, GAD 65, in young diabetes-prone animals (Kaufman, Clare-Salzler, Tian, Forsthuber, Ting, Robinson, Atkinson, Sercanz, Tobin and Lehmann 1993; Tisch, Yang, Singer, Liblau, Fugger and McDevitt 1993). This suggested that GAD may be a major β-cell autoantigen and was consistent with the finding that in GAD antibody positive patients, GAD is a

target of both autoantibodies and T-cells (Eisenbarth, Gianani, Pugliese, Verge and Pietropaola 1994; Todd and Bain 1992). GAD antibodies increase with age (Jacob, Peterson, Moody and Allan 1994) and by the time of diagnosis about 75-80% of patients are GAD positive (Baekkeskov et al. 1990, Atkinson and Maclaren 1990). GAD antibodies are more prevalent in people with certain high risk major histocompatibility (MHC) class II genes such as HLA-DR3/4 and HLA-DQβ1 (Jacob et al. 1994; Serjeantson, Court, Mackay et al. 1993). Therefore, in addition to disturbances in T cell-mediated immunity, IDDM patients often have several autoantibodies present.

Genetic and Environmental Factors

Expression of MHC class I and class II antigens in the islet may be involved in the pathogenesis of IDDM and the expression of these molecules particularly class I, on the islet cell surface, probably sets in motion a series of events that leads to the destruction of the islet β cells. However, the genetic susceptibility to IDDM is mediated only in part by genes in the human leukocyte antigen (HLA) class II region that either predispose or protect people from developing the disease (Todd, Bell and McDevitt 1987). HLA-DR3 and/or DR4 are associated with increased risk to develop the disease, while haplotypes with HLA-DR2 protect against type I diabetes. These HLA genes operate as susceptibility factors not as determinants, in that the majority of patients with these genes do not develop IDDM (David, Leslie and Elliott 1994).

Studies in identical twins show a concordance rate of only ~35% for IDDM indicating that even the most genetically susceptible individuals do not necessarily develop IDDM (Barnett, Leslie and Pyke 1981; Olmos, A'Hern, Heaton, Millward, Risley, Pyke and Leslie 1988). This suggests that genetic predisposition and environmental factors are both important in the etiology of the disease (Scott, Elliott and

Kolb 1989a).

Another line of evidence for the importance of environment in IDDM emerges from epidemiological studies. The geographic variations in the incidence of IDDM seem to some extent to reflect the differential distribution of genetic factors associated with susceptibility or resistance to IDDM between populations (Dorman, Laporte, Stone and Trucco 1990). However, several features in the geographic and temporal patterns of IDDM underline the important role of the environment (Diabetes Epidemiology Research International Group 1990, Lévy-Marchal et al. 1995). Also, the fact that most new cases of IDDM are in individuals with no family history of the disease further suggests an important effect of environmental factors on expression of diabetes. Furthermore, the rapidly increasing incidence of IDDM in several countries strongly suggests that environmental factors must have a major effect on the disease process.

Environmental factors considered in the etiology of type I diabetes have been chemical toxins, stress, diet and infectious agents (Scott 1994a). Congenital rubella is a rare cause of diabetes and other viral infections during pregnancy could be important risk determinants for some cases of IDDM. The environmental effect that causes IDDM operates over a limited period in childhood (David et al. 1994). Virus infection may directly kill β cells in a specific manner depending on the type of virus and the species and strain of host (Yoon and Bachurski 1984).

However, recent studies using PCR analysis did not find diabetogenic viruses in the pancreas of newly diagnosed patients (Buesa-Gomez, de la Torre, Dyrberg, Landin-Olsson, Mauseth, Lernmark and Oldstone 1994; Foy, Quirke, Williams, Lewis, Grant, Eglin and Bodansky 1994). It is known that diabetes-prone BB rats and NOD mice have the highest diabetes incidence when maintained in SPF facilities (Rabinovitch 1994).

The difficulties in identifying diabetogenic infectious agents in humans, NOD mice and BB rats and the finding that diabetes occurs spontaneously in BBdp rats raised in a germ-free environment (Rossini, Williams, Mordes, Appel and Like 1979), have focused even further attention on the possibility that dietary components may be the main environmental factors required in diabetes pathogenesis. Approximately 80% or more of the diabetes which occurs in the genetically predisposed BB rat and NOD mouse can be significantly delayed or prevented by manipulation of diet.

Animal Models

The direct study of food diabetogens has only recently been possible due to the availability of two animal models of IDDM: the diabetes-prone Bio-Breeding (BBdp) rat (Nakhooda, Like, Chappel, Murray and Marliss, 1977; Crisa, Mordes and Rossini, 1992) and the non-obese diabetic (NOD) mouse (Kikutani and Makino, 1992). A majority of BB rats and NOD mice spontaneously develops a disease that shares several immunological and genetic features with human IDDM (Castaño and Eisenbarth 1990). In all three species, mouse, rat and human, it is genes in the MHC class II region that impart the major risk of developing diabetes. BBdp rats were used for the studies described in this thesis.

Diabetes in the BBdp rat is autoimmune-based and resembles human type I diabetes, appearing equally in males and females around puberty and adolescence between 55 to 120 d, with a mean age at onset of about 90 d. Development of overt diabetes is sudden, and is associated with weight loss or failure to gain weight, increased blood glucose, glucosuria, and ketonuria.

The presence of autoantibodies against β-cell components has been reported as early as 40 d and mononuclear cells can be seen in and around the islets beginning at

about 50 d. By about 70 d, these leukocytes can be seen inside the islet and this is called, insulitis. Several lines of evidence indicate that the disease occurs spontaneously as the result of interaction among susceptibility genes and environmental factors (Rossini, Greiner, Friedman and Mordes 1993).

A mononuclear cell infiltrate is often also observed in the thyroid. BBdp rats display lymphopenia which appears to be necessary for most diabetes development (Jacob, Petterson, Wilson, Mao, Lernmark and Lander 1992). Although human patients do not display such a lymphopenia, there are a few reports of mild lymphopenia in newly diagnosed patients (Kaaba and Alhardi 1995; Peakman, Warnock, Vats, McNab, Underhill, Donaldson and Vergani 1994) and induced lymphopenia in rodents, is also linked to anti-islet immunity and diabetes (Penhale, Stumbles, Huxtable, Sutherland and Pethick 1990; Fowell and Mason 1993). Both diabetes incidence and pancreatic inflammation (insulitis) in these animals are affected by diet (Scott and Kolb 1996b).

Diet and Diabetes

Evidence from our laboratory, first published in the early 1980's, reported prevention of diabetes in BBdp rats by feeding a semipurified, casein-based diet that was a modification of the American Institute of Nutrition recommended diet for rats and mice, AIN-76A (Scott and Trick 1983; Scott, Trick, Hynie, Braaten, Nera 1984; Scott, Mongeau, Kardish, Hatina, Trick, Wojcinski 1985). Reports from several laboratories support the suggestion of a major contribution of diet in spontaneous diabetes (Elliot and Martin 1984; Issa-Chergui, Guttmann, Seemayer, Kelley and Colle 1988; Hoorfar, Buschard, Brogren 1992; Li, Scott, Park, Yoon 1995). Other epidemiological data and studies in BBdp rats suggested a role for early exposure to cow milk in the etiology of IDDM. The first observations of a possible association between diet and IDDM in

humans were from epidemiological studies on infant feeding patterns (Borch-Johnsen, Mandrup-poulsen, Zachau-Christiansen, Joner, Christy, Kastrup and Nerup 1984) which led to the "milk hypothesis" linking early exposure in infancy to cow milk as the triggering event in IDDM (Karjalainen, Martin, Knip, Ilonen, Robinson, Savilahti, Åkerblom and Dosch 1992). The basis of the milk hypothesis has been challenged (Ellis and Atkinson 1996; Scott, Norris and Kolb 1996a; Norris, Beaty, Klingensmith, Yu, Hoffman, Chase, Erlich, Hamman, Eisenbarth and Rewers 1996; Shatz and Maclaren 1995) and it has been suggested that certain plant foods (wheat and soy) may be more important (Scott et al. 1996a; Scott and Kolb 1996b).

It is not known how food antigens might induce IDDM but the mechanisms are becoming clear. For example, there is evidence that diet affects diabetes outcome in the BBdp rat by somehow increasing expression of MHC class I molecules on the surface of target β-cells, possibly as early as weaning (Li et al. 1995). There are also later effects indicating diet can change the predominance of islet inflammatory cells from Th1 to Th2 (Scott et al. 1996a; Scott, Cloutier, Kleemann, Woerz-Pagenstert, Rowsell, Modler and Kolb In press, 1997). These changes in the target beta cell membrane and the immune system may be important early events in the autoimmune process (Scott 1994b) and further support the idea that diet is a major controlling factor in diabetogenesis.

Results from our laboratory (Scott and Trick 1983; Scott et al.1985; Scott 1994b; Li et al. 1995; Scott 1995a; Hoorfar, Scott and Cloutier 1991; Scott, Sarwar and Cloutier 1988; Behrens, Scott, Madere and Trick 1984; Behrens, Scott, Madere, Trick and Hanna 1986) and other laboratories (Elliott and Martin 1984; Issa-Chergui et al. 1988; Hoorfar et al. 1992; Atkinson, Winter and Skordis 1988) show that, compared to standard plant-based laboratory diets, certain individual sources of amino acids when fed in modified,

semipurified diets, inhibit development of diabetes in BBdp rats. The range of the diabetes-inducing potential of diets containing different food protein sources is remarkably wide. For example, feeding diets in which the only amino acid source is a commercial mixture of caseins or hydrolysed caseins results in diabetes incidence (%) of 11±6 (14 expts, n=533 rats) and 13±7 (10 expts, n=227 rats) respectively, whereas the NIH-07 (NIH, a standard rodent diet, open formula, Bieri et al. 1977), cereal-based diet caused 68±7 (7 expts, n=164 rats) of BBdp rats to become diabetic. Cereal based rodent diets are the most diabetogenic and several other amino acid sources including casein, hydrolyzed casein (HC), lactalbumin, hydrolyzed lactalbumin, fish meal, corn meal and other diets are the least diabetogenic (Scott 1994a). Therefore, it is changes in the source of dietary amino acids that have the most effect on diabetes outcome in BBdp rats (Scott and Trick 1983; Scott et al. 1984; Scott et al. 1985; Elliott and Martin 1984) and diet is responsible for approximately 80% of the diabetes that appears in groups of BBdp rats. Among semi-purified diets containing various protein sources, soybean meal and wheat gluten are the most consistently diabetogenic (Scott, Cui and Rowsell 1994), displaying moderate to strong diabetogenicity. It has been suggested, but not proven, that these food diabetogens are proteins or peptides which contain sequences that are similar to antigens associated with the insulin secreting beta cells. However, the exact molecular identity of these diabetes inducing agents is not known. The main focus of this thesis is the identification of potential diabetogens in soybean products.

The NIH diet is mainly a cereal-based mixture in which wheat and soy fractions make up 45% of the diet and together account for 43% of the protein. Fish meal, which is nondiabetogenic (Scott et al. 1988), accounts for 27% of protein, and 8% comes from milk powder. The remaining protein comes from corn, yeast and alfalfa, all of which

probably contribute little to the diabetogenicity of the NIH diet. Results from our laboratory showed that the diabetogenicity of skim milk powder was too variable to explain the consistent diabetogenicity of cereal-based diets. Milk is a small part of the NIH diet and when tested as the sole source of amino acid in semipurified diets, displays a diabetogenic potential that is mild, highly variable and secondary to the more potent and consistent plant food diabetogens in wheat and soy (Scott 1994b, Scott, 1996). Therefore, the NIH diet is associated with a high diabetes frequency in BBdp rats and is used as a positive control diet in our feeding studies. Soy is a major ingredient of virtually all laboratory rodent diets and it is consumed in a variety of formulated foods in the human diet such as flours, soy milks, tofu, protein mixtures, dairy and egg fractions and soy-based infant formulas.

Soy and Diabetes

Little is known about soy and IDDM in humans, but there are indications that it can be diabetogenic. Soybean meal, flour or flakes all produce diabetes frequency of about 50% and insulitis frequency of at least 57% to 80% (Scott 1994a) in BBdp rats. In another experiment, (Hoorfar et al. 1991) soybean meal resulted in the highest diabetes frequency, 47%, among the test diets, which is in agreement with two previous reports with diabetes frequency at 60% (Brogren, Hoorfar and Buschard 1989) and 38% (Atkinson et al. 1988). These results indicated that certain soybean meals and other sources of soy proteins can promote diabetes in BBdp rats when fed in purified diets (Scott and Marliss 1991; Scott 1996).

In several previous experiments in our laboratory, diabetes-prone BB rats fed a defined diet in which the sole source of protein was soybean meal showed a mean diabetes frequency of $47\pm7\%$ (7 expts, n=117 rats) ~4 times higher than that observed when the

usual negative control diets with casein or hydrolyzed casein (HC) were fed. There is one report that IDDM patients were more likely to have been fed soy-based formulas than controls (Fort, Lanes, Dahlem et al. 1986) and that patients with another organ specific autoimmune disorder, thyroiditis, were also more likely to have been fed soy-based formulas (Fort, Moses, Fasano et al. 1990). Soybean meal is a less refined preparation unlike the soy protein isolates (SPI) used in soy-based infant formulas. Previous results in our laboratory indicated that diets containing SPI were less diabetogenic in BB rats than soybean meal diets; an SPI-based diet produced 33% diabetes incidence and 25% of animals fed an SPI-based infant formula became diabetic. However, the rate and final diabetes outcome in soymeal or SPI-fed BB rats was greater than 2 SD above the mean of negative control, hydrolyzed casein diet (Scott, 1996).

Various soy sources were partially characterized in our laboratory using soy preparations treated with ethanol, papain, hexane and other chemicals and fed to BB rats (Scott 1996; Scott 1994a; Hoorfar et al. 1991). Results indicated that the diabetogenicity of soybeans was unrelated to the Kunitz trypsin inhibitor, heat stable, unaffected by hexane or ethanol extraction, and was decreased but not abolished by hydrolysis with papain. Therefore, the diabetogenic activity was not related to a hexane soluble lipid or soybean trypsin inhibitor (2S globulin protein) or ethanol soluble phytoestrogen. It may be a heat stable, globulin protein or peptide whose activity is decreased by papain hydrolysis. Thus, soybeans contain a material, likely a protein or peptide, that produces moderate to high diabetes frequency in BBdp rats (Scott, 1996).

Soy Proteins

Soybean proteins consist of two major fractions, the globulin fraction (containing the 15S, 11S, 7S and 2S proteins) which makes up 85% of total protein, and the whey

fraction (Lusas and Riaz 1995, Burks, Brooks and Sampson 1988). The 2S fraction is 10-20 % of the total protein and includes many small proteins such as trypsin inhibitors and cytochrome C. The 7S fraction accounts for 30-40% and is composed mainly of the 7S globulin (conglycinin) but also includes some minor proteins such as hemagglutinin, lipoxygenases, and amylases. The 11S fraction also makes up 30-40 % of the total and includes principally the 11S globulin (glycinin), and the 15S fraction represents 10-15 % of the total, comprising mainly aggregates of the 7S and 11S proteins (Than and Shibasaki 1976). The conglycinin and glycinin fractions are the major constituents of commercial soy protein isolate products.

Although raw (unheated) soy products cause pancreatic exocrine cell hyperplasia (Liener and Hasdai 1986, Liener, Goodale, Deshmukh, Satterberg, Ward, DiPietro, Bankey and Borner 1988), the products tested in our laboratory have all been heat treated. There are potential connections between heat treated soy proteins (soybean meal, soy protein isolates, soy infant formulas) and the endocrine pancreas. A polypeptide of molecular mass 37 kDa has been purified as a bile acid binding component of soy protein isolate. Interestingly, this protein potentiates the *in vitro* inhibitory action of insulin on fat decomposition that was stimulated by epinephrine in rat adipose cells (Makino, Nakashima, Minami, Moriyama and Takao 1988). The major insulin modulating components of soybean protein are the acidic A1a and A2 polypeptides of glycinin. Another bile acid binding protein of molecular mass 40,000 (Minami, Moriyama, Kitagawa and Makino 1990) isolated from pea seeds also modulates insulin action. Two proteins from soybeans, designated Si 30 and Si 60, have been shown to have homologies with insulin and are insulin binding proteins (Barbashov et al. 1991).

Immune Response to Dietary Proteins

Ingested proteins are hydrolyzed into their constituent amino acids in the proximal gastrointestinal tract by acid and enzymatic hydrolysis. However, small amounts of orally administered proteins escape digestion and are absorbed as antigenic proteins or peptides. This may have varying immunological consequences. More rarely, a systemic immune response may appear, including antibody production and activation of antigen-specific T-cells. Finally, after repeated exposure to an antigen, systemic immunological tolerance to the antigen may develop (Mowat 1987). Age at the time of exposure and dose of the dietary protein is important in determining the nature of the systemic response. The intestinal epithelium in human is permeable to protein macromolecules during the first months after birth (Kuitunen, Savilahti and Sarnesto 1994). At this age, after oral exposure to an antigen, systemic sensitization is easily induced. Studies in infants have demonstrated that feeding cow milk soon after birth leads to systemic production of cow milk protein antibody production with a peak IgG antibody level at 3 months of age. After this initial peak, the IgG response slowly declines with age even with repeated exposure (Kletter, Gery, Freier and Davies 1971a).

Suppression of immune responses following orally fed protein antigens, a process called oral tolerance, has long been recognized in laboratory animals (Wells 1911). Studies in mice have shown that feeding of an antigen during the early neonatal period leads to priming of the immune system, while feeding at a later age induces tolerance (Strobel and Ferguson 1984). Schur et al. found that IgG subclass proteins can be detected as early as 11 weeks in the fetus (Schur, Alpert and Alper 1973). By about 33 weeks, levels in the fetus were very similar to those in the mother (Morell, Skvaril and Baramdun 1976).

The first observation linking exaggerated immune response to a dietary protein to insulin dependent diabetes was provided by Beppu, Winter, Atkinson, Maclaren, Fujita and Takahasha (1987), who showed that NOD mice had higher levels and higher frequency of positivity of serum IgG, IgA and IgM antibodies to BSA than did control mice, as measured by ELISA. Furthermore, diabetic NOD mice had a higher frequency of IgG antibodies to BSA than did non-diabetic NOD mice. Savilahti, Åkerblom, Tainio and Koskimies (1988) found in children with newly diagnosed IDDM, increased levels of IgA antibodies to whole cow milk and β-lactoglobulin (BLG), and IgG antibodies to BLG. Also, Karjalainen et al. (1992), found elevated levels of IgG antibodies to BSA in 100% of the 142 Finnish children they studied with newly diagnosed IDDM compared with only 4% of control children. This result has been difficult to confirm (Atkinson, Bowman, Kao, Campbell, Dush, Shah, Simell and Maclaren 1993).

Dreau, Lallés, Philouze-Romé, Toullec and Salmon (1994) found that immunoblotting patterns of raw soybean with sera from early-weaned pigs (28 day old) showed two bands (22 and 36 kDa) recognized by IgA and IgM antibodies, respectively. The observed IgA response could be related to the predominance of this immoglobulin isotype in the digestive tract (Mestecky and Mc Ghee 1987) whereas the presence of IgM anti-soybean antibodies suggests a primary antibody response. Also an increase in circulating specific antibodies, especially IgG, was shown in early weaned pigs (Li et al. 1990). The molecular mass of soy protein fractions ranges from 8 to 600 kDa and is certainly large enough to stimulate antibody production and larger than most, if not all, of the nutritional proteins found in cow milk (Anglemier and Montgomery 1976). There are no reports linking directly immune response to dietary soy proteins with type I diabetes in humans or animals.

Early Infant Feeding and Oral Tolerance

The timing of introduction and duration of feeding of certain amino acid sources may be important but this is not yet clearly established in susceptible humans. The role of early exposure to cow milk protein and breast feeding is the subject of much controversy. Work from our laboratory (Scott and Rowsell 1995) using BBdp rats and studies by others of humans (Atkinson 1993) with various autoimmune diseases indicate that normal development of oral tolerance may be disturbed in susceptible individuals. This is shown by the presence of antibodies to a variety of food antigens encountered before and after weaning.

Kostraba, Cruickshanks, Lawler, Jobin, Rewers, Gay, Chase, Klingensmith and Hamman (1993) found that exposure to solid foods by 3 months of age was associated with a 2.5 fold increased risk of IDDM. Alternatively, early cessation of breast-feeding is associated with the early introduction of foreign antigens, such as cow milk proteins (Gerstein 1994; Kostraba et al. 1993; Virtanen, Räsänen, Ylönen, Aro, Clayton, Langholz, Pitkäniemi, Savilahti, Lounavao, Toumilehto and Åkerblom 1993). In one recent study, the attributable risk was 8% for cow milk and 25% for solid foods if these factors were introduced after 3 months of age (Kostraba et al. 1993). In addition, in the manufacture of infant formulas, there are large variations in heat-treatment of the products, resulting in differences in solubility and digestibility of the proteins in the formulas (Rudloff and Lonnerdal 1992). Heat-treatment may, in fact, be profoundly important in determining the immune response following feeding of milk products. Also recently, the report of the American Academy of Pediatrics (AAP) Work Group on Cow Milk Protein and Insulin-dependent Diabetes Mellitus stated that early exposure to cow milk protein may be an important factor in the initiation of the β-cell destructive process

in some individuals and recommended that with the exception of cow milk-based infant formulas, high risk infants not be fed products containing cow proteins during the first year of life. In addition, the feeding of soy-based formulas was discouraged based on studies reported from our laboratory.

The timing and quantity of exposure to a dietary antigen may be the most important elements in determining the ensuing immune response. However, the specific molecular characteristics of the proteins also are of significance. Protein fractions of human and non-human milk are perhaps the most widely investigated dietary proteins, largely because of the key role of milk as a source of energy and nutrients during the first years of life. Because oral immune tolerance is generally ascribed to protein antigens, the ability to affect diabetogenesis in BBdp rats by oral dosing with food diabetogens in early infancy, also suggests the protein contains the active ingredient (Scott, Rowsell 1995).

Soy Foods and Other Related Diseases

Soy products are major constituents of rodent laboratory diets and are gaining increased application for the past two decades in Western cultures and diets. Soy formula is a commonly used alternative to cow milk formula and is fed to an estimated 25% of all formula-fed infants in the United States. Soy protein formulas are used for different conditions, including cow milk protein allergy, lactose and galactose intolerance, and severe gastroenteritis. Digestive intolerance to food proteins may occur in childhood as a result of a wide range of pathophysiologic mechanisms.

There are two types of food sensitive enteropathy, permanent and temporary. The food sensitive enteropathies of early childhood belong to the latter group and these disorders are temporary and may follow gastroenteritis. The reason why these enteropathies are temporary has not yet been established. There is growing evidence that

soy proteins can induce enteropathy in young infants with and without cow milk intolerance, with atrophy of the villi similar to that caused by cow milk protein intolerance (Ament and Rubin, 1972; Halpin, Byrne, Ament, 1977; Powell, 1978; Merrit, Carter, Haight and Eisenberg, 1990).

Food allergy and food intolerance may be confused because both produce similar symptoms, especially in young children with clinical manifestations of food allergy localized to the gastrointestinal tract. On the other hand, food sensitive enteropathy may be defined as the food-related clinical syndromes associated with an abnormal small intestinal mucosa. Whatever the mechanisms, digestive intolerance to food proteins with or without enteropathy is primarily a temporary condition of infancy, in contrast to most forms of food allergy. In children with these disorders, symptoms usually resolve by 1 to 3 years of age. Some risk factors for the development of these conditions appear to be early exposure to cow milk, acute infectious diarrhea, and malnutrition. Macromolecular absorption of potential antigens across the mucosal barrier, intestinal antigen binding by secretory IgA, and the local cell-mediated immune system are important factors in the development of an immune response to food antigens (Walker, 1986; Munch, Pelletier, Walker, 1989).

The soy proteins used in infant formulas appear to be equally and, in some cases, more antigenic than the heat treated cow milk based formulas (Witherly 1990). Eastham et al., (Eastham, Lichauco, Grady and Walker, 1978) concluded that soy protein is at least as antigenic as cow milk protein and should be used with caution. The large molecular mass and multiple protein subclasses of soy proteins rival the 20 to 30 antigenic fractions of bovine milk. Burks et al. (Burks, Brooks and Sampson 1988) using ELISA to investigate soy-sensitized infants with atopic dermatitis, demonstrated allergen-specific

IgG and IgE antibodies to the 7S, 11S, and whey protein fractions of soy extracts. The fact that soy proteins are diabetogenic when fed to BBdp rats (and NOD mice, Hoorfar et al. 1993), provided an accessible and controlled model system for further identifying which soy protein bands may be diabetes related.

Questions Posed and Approaches Used in this Study

The aims of this study were to identify and partially characterize diabetes-related soy proteins using immunoblotting with sera from (i) BB rats at different levels of risk for development of diabetes, BBc (non-diabetes prone), BBdp (diabetes-prone) and BBd (overt diabetic) and (ii) newly diagnosed diabetic children.

The major questions posed in this study were as follows:

(1) Are there unique soy proteins that are identified using immunoblotting of soy protein extracts with sera from BB diabetes-prone and diabetic BB rats?

(2) Is the timing of appearance of these antibodies in blood related to development of diabetes in cross sectional and prospective samples?

(3) Can early oral dosing with soy antigens protect diabetes-prone BB rats from becoming diabetic?

(4) Can the results from studies in BBdp rats help to identify soy proteins that are potentially diabetes-related in human patients?

(5) Are there differences in immunoreactivity of different soy materials and is this related to diabetes outcome?

(6) Do soy antibodies cross react with (RIN) β-cell antigens?

Several approaches were used to answer these questions. First, Western blots of soy protein extracts were probed with sera from BB rats with different risk of developing IDDM to identify potential diabetes-related soy immunogens. The development of soy

antibodies with age was investigated and the diabetogenicity of various SPI-based infant formulas was determined. Using the approach developed in the BB rat studies, Western blots of soy proteins were performed using sera from control and newly diagnosed children with diabetes. The effect of early oral exposure to soymeal on diabetes development in soymeal-fed BBdp rats was determined and the associated effects on diabetes related soy antibodies were measured. The potential cross-reactivity of soy antibodies with β-cell antigens was examined by pre-absorbing BB rat sera with a rat β-cell tumour line, RINm5F cells.

The approach we used in this study is similar to that used by several investigators attempting to identify soy, peanut and other food antigens involved in food allergies and is also similar to the approach used to identify β-cell autoantigens such as GAD. We believe this study is the first to identify potential soy food diabetogens. It is hoped that the results of this study will lead ultimately to the chemical identity of these agents.

MATERIALS AND METHODS

Animals

Rats used in these studies were provided by the Animal Resources Division of Health Canada. This colony is maintained by mating sibs, usually non-diabetic female with diabetic male and diabetes incidence has remained between 50-60% for the past 12 years. Rats are monitored twice weekly and diabetes is diagnosed based on the appearance of glucosuria, excess urine output, failure to gain weight and fasting blood glucose ≥11.1 mM. Diabetic rats are implanted with a sustained release insulin implant (½ to 1 per rat) containing 14% bovine insulin and 86% palmitic acid with a release rate of ~2 U/24 h for up to 40 d (Linplant, Linshin Inc, Scarborough, Ontario). A colony of non-diabetes-prone rats, which never develop diabetes, was derived from the original colony and these animals are referred to as BB control (BBc) rats. Both colonies are kept in rooms with filtered air and specific pathogen-free (SPF) environment. The animals are routinely tested and found to be antibody-free with respect to Sendai virus, pneumonia virus of mice, rat caronavirus/sialodacryoadenitis virus, Kilham rat virus, Toolan's H-1 virus, reovirus type 3 and mycoplasma pulmonis.

Animal maintenance, testing for diabetes and prospective blood samples were provided by Jocelyn Souligny, Animal Resources Division of Health Canada. Animals were maintained in banks of 30 or 60 stainless steel, wire bottom cages and permitted free access to food and water. Pups were weaned at about 23 d onto appropriate test diets which were fed in meal form in aluminium bowls up to age 150 d. Rats were weighed weekly between the ages of 30 d and 100 d. After 55 d of age, rats were tested twice weekly for glucose in the urine (Testape, Eli Lilly, Toronto, Ontario). Those rats with a value of ≥2+ were fasted overnight and blood glucose was measured in the morning with

a glucometer. Animals with blood glucose ≥11.1 mM were killed by exsanguination under 3% isoflurane or halothane in oxygen anaesthesia.

Diets

A. Control Diets

Hydrolysed Casein (HC)-Negative Control

The protective, negative control diet, based on hydrolysed casein (HC) as sole amino acid source, was a modification of the American Institute of Nutrition recommended diet for rats and mice, AIN-76A (Bieri, Stoewsand, Briggs, Phillips, Woodard, Knapka 1977; Bieri, Stoewsand, Briggs, Phillips, Woodard, Knapka 1980). Hydrolysed casein was purchased as a commercial product, Pancase S, from Champlain Industries, Mississauga, Ontario. The HC diet contained 20% hydrolysed casein, 53% corn starch, 12% sucrose, 5% corn oil, 5% cellulose-type fiber (Solka-Floc), supplemented with 1% AIN-76A (Bieri, Stoewsand, Briggs, Phillips, Woodard, Knapka 1980) vitamin mix (ICN Biochemicals, Cleveland, Ohio, U.S.A.), 3.5% mineral mix, choline bitartrate, 0.2% and DL-methionine, 0.3%.

NIH-Cereal-Based, Positive Control

The positive control, diabetogenic diet was a standard, open formula (percent of each ingredient known), cereal-based laboratory rodent diet called NIH-O7 (NIH; Zeigler Brothers, Gardner, PA). The NIH diet is a mainly cereal-based mixture composed of 82.5% plant materials. The components include: 5% dried skim milk, 10% fish meal, 12% soybean meal, 4% alfalfa meal, 3% corn gluten meal, 24.5% ground corn, 23% ground hard winter wheat, 10% wheat middlings, 2% Brewer's yeast, 1.5% molasses, 2.5% soybean oil, 0.5% sodium chloride, 1.25% dicalcium phosphate, 0.5% ground limestone, 0.25% premixes (Bieri, Stoewsand, Briggs, Phillips, Woodard, Knapka 1977;

Knapka, Smith, Judge 1974).

Purina 5001

Laboratory rodent Purina 5001 diet is a closed formula (ingredients known but percent of each not given) rodent diet recommended for rats, mice and hamsters. This diet is a mixture of ground yellow corn, soybean meal, dried beet pulp, fish meal, ground oats, brewers' dried yeast, alfalfa meal, cane molasses, wheat germ meal, dried whey, meat meal, wheat middlings, supplemented minerals and vitamins. This diet is composed of 23.4% protein, 4.5% fat, 5.8% crude fiber, 7.3% ash and 2.5% added minerals.

B. Semipurified Test Diets

The semipurified test diets were modifications of the American Institute of Nutrition recommended diet for rats and mice, AIN-76A (Bieri et al. 1977 and 1980), and were fed in powdered form in individual metal bowls.

Soybean Meal

The soybean meal diet was made up with: 41.4% soybean meal (Ritchie's Feed, Ottawa, Canada), 37.1% corn starch, 12% sucrose, 4.2% corn oil, 3.5% AIN-76A mineral mix and 1.0% AIN-76A vitamin mix, 0.2% choline bitartrate, 0.6% DL-Methionine (ICN).

Prosobee Powder Infant Formula

Prosobee powder infant formula diet was made up with: 93% Prosobee powder (Mead Johnson, Belleville, Ontario, Canada), 4% casein hydrolysate (Champlain Industries, Mississauga, Ontario, Canada), 3% alphacel (cellulose type fiber, Solka-Floc, Teklad, Madison, WI).

Prosobee Liquid Infant Formula

Prosobee liquid infant formula diet was fed as purchased Prosobee concentrated

liquid formula (Mead Johnson, Belleville, Ontario, Canada), ~60 ml/d of the formula were fed in pre-sterilized glass bottles attached to the side of the cage.

Soy Protein Isolate

Soy protein isolate (SPI) diet was made up with: 20.7% soy protein isolate (ICN Biochemicals), 52.8% corn starch, 12% sucrose, 4.2% corn oil, 5.0% alphacel (cellulose type fiber), 3.5% AIN-76A mineral mix and 1.0% AIN-76A vitamin mix, 0.2% choline bitartrate, 0.6% DL-methionine.

Hydrolysed Soy Protein Isolate

Hydrolysed soy protein isolate diet was made up with: 20% N-Z Soy Peptone (Sigma P-1265, St.Louis, U.S.A.), 52.7% corn starch, 12% sucrose, 5.0% corn oil, 5.0% alphacel, 3.5% AIN-76A mineral mix and 1.0% AIN-76A vitamin mix, 0.2% choline bitartrate, 0.6% DL-methionine.

Serum Samples

A. Rat Serum Samples

Blood was either collected at necropsy from anaesthetized animals by exsanguination from the abdominal aorta or by bleeding from the orbital plexus of the eye. Blood from 5 and 15 day old pups was collected following decapitation and samples from entire litters were pooled to obtain sufficient serum. All blood was put into serum separation tubes (Vacutainers, Becton-Dickson, Rutherford, New Jersey), centrifuged for 20 minutes at 1000×g, 4°C and serum was transferred to 0.5 ml Eppendorf tubes in 300μl aliquots and stored at -20°C until used.

B. Human Subjects

Human serum samples were kindly supplied from newly diagnosed diabetic children from two sources:

1) Dr. K.A. Faught, Pediatrician at Children's Hospital of Eastern Ontario (CHEO, Ottawa, Ontario).

   The following information was obtained: name, date of birth, sex, date of onset of symptoms, recent infection, family history, date of blood sample, fasting blood glucose and nature of any treatment.

2) Dr. O. Simell, Professor and Chairman, Department of Paediatrics, University of Turku, Finland.

   Information provided for each patient was as follows: name, sex, blood glucose before insulin treatment, blood glucose a day after initiation of insulin treatment, diabetes in relatives of the child, preceding infections and HLA-haplotype.

Food Protein Extracts

Soy proteins were extracted in 0.03 M Tris-HCl (Sigma T-3253, St.Louis, MO., U.S.A.) buffer containing 0.01 M mercaptoethanol at pH 8.0 at a ratio of food material : buffer of 1 :40 (Thanh, Okubo and Shibasaki 1975). This mixture was stirred for one hour at room temperature, centrifuged for 10 minutes in an Eppendorf microcentrifuge at about 12000 $\times$g, the supernatant was removed and aliquots of 100μl were frozen.

Protein Assay

Protein concentration in the extracts was assayed using the Bradford protein micro assay as adapted by BIORAD for 96 well plates (Bredford et al., 1976; Microplates, Nunc Immunoplate Maxisorp C96). BSA standard dilutions were made in a range of 0-24 μg protein in 1 ml volumes of 100 μg/ml of stock BSA and 200 μl BIORAD protein reagent. Sample extracts were also made in a 1 ml volume: 5 μl of extract, 200 μl BIORAD protein reagent and 795 μl distilled water. After 20 minutes incubation of samples with BIORAD dye reagent at room temperature, 250 μl/well of each standard dilution was

pipetted in triplicate and 250 μl/well of each sample extract was pipetted in 8 replicate wells. Absorbances were read at 595 nm with 490 nm as reference in a microplate reader (BIORAD, model 3550-UV). Blank samples were run with sample buffer and this absorbance was subtracted from the sample absorbance values. The protein concentration was then calculated from the equation of the standard curve.

SDS-PAGE, Continuous 1D Gel Electrophoresis

Protein samples were run in a Mini-Protean II Dual Cell BIORAD apparatus after casting the separation and stacking gels. The separation gel consisted of 5 ml solution C [20 gm acrylamide 99.9% (BIO-RAD #161-0101, Hercules, CA.), 0.26 gm bis-acrylamide (N,N'-methylene-bis-acrylamide, BIO-RAD #161-0201) in 100 ml distilled water] plus 2.5 ml solution A [48 ml 1N HCl (Baker Analyzed), 36.3 gm Trisbase (Sigma 7-9, T-1378, St.Louis, MO., U.S.A.), 0.23 ml TEMED (N,N, N',N'-Tetra-methylethylenediamine, BIO-RAD #161-0801) in 100 ml distilled water] and 2.3 ml distilled water. After de-gassing, 100 μl of 10% SDS (sodium dodecyl sulfate, Sigma L-3771) and 100 μl of 10% ammonium persulfate (BIO-RAD #161-0700) were added. The stacking gel was a combination of 1.25 ml solution C and 0.95 ml solution B [48 ml 1N HCl, 5.98 gm Trisbase (Sigma 7-9, T-1378), 0.46 ml TEMED in 100 ml distilled water] plus 5 ml distilled water. After de-gassing, 100 μl 10% SDS and 100 μl of 10% ammonium persulfate were added.

Based on the results of the protein assay, 30 μl of different diluted proteins were loaded in each lane (~25μg/lane) which was a 1:1 dilution in sample loading buffer [1000 μl of 10% SDS, 500 μl of 1M Tris-HCl (Sigma T-3253), 200 μl of 100mM PMSF (Phenylmethylsulfonyl fluoride, Sigma P-7626), 100 μl of 100 mM EDTA (ethylenediamine tetra acetic acid, Sigma ED2SS), 1.26 gm glycerol (Sigma G-8773),

1700 μl distilled water, 500 μl mercaptoethanol (Sigma M-7154)] was loaded in different lanes along with a lane of molecular weight markers (kaleidoscope prestained standards, BIO-RAD # 161-0324), as well as a soy protein isolate (SPI) lane to be used as a control for each gel or blot. Gels were run in running buffer [3 gm Trisbase, Sigma 7-9 T-1378; 14.2 gm glycine, Fisher Biotech BP 381-5; 10 ml of 10% SDS Sigma L-3771; 100 μl of PMSF, Sigma P-7626 in 1 litre distilled water] at 180 V, 109 mA for one hour using an LKB 2197 Electrofocusing Constant Power Supply.

Silver Staining of the Gels

The silver staining protocol was adapted from Blum, Beier and Gross (1987). Gels were incubated in fixing solution [40% methanol, 10% acetic acid in distilled water] for two hours. Three 20 minute washes in 30% ethanol were carried out. The reduction step involved incubating 1 minute in thiosulfate reagent [0.02% sodium thiosulfate in ddH_2O, British Drug House, Toronto] followed by three 20 second washes in distilled water. Gels were incubated 20 minutes in silver nitrate reagent [0.2% silver nitrate, Sigma S-7276; 0.02% formaldehyde (37%) in distilled water] followed by three 20 second washes in distilled water. Gels were then incubated 3-5 minutes in developer [3% sodium carbonate, British Drug House, Canada; 0.05% formaldehyde; 0.0005% sodium thiosulfate in distilled water] followed by three 20 second washes in distilled water. They were then incubated for 5 minutes in stop reagent [0.5% glycine, Fisher Biotech] and washed 2×30 minutes in distilled water.

Coomassie Stain for Gels

Gels were incubated in Coomassie Blue solution [25 ml isopropanol, 10 ml glacial acetic acid (Baker Analyzed), 5 ml stock Coomassie Blue (1.0% Coomassie Blue R250 in methanol, LKB 1840-101) in 60 ml distilled water] for one hour with agitation.

Destaining of gels was carried out with multiple changes of destaining solution [125 ml isopropanol, 50 ml glacial acetic acid in 325 ml distilled water] for one hour and finally placing the gel in preserving solution [50 ml glycerol, Sigma G-8773 in 450 ml distilled water].

Transferring (Blotting)

Proteins were transferred to Protran nitrocellulose membranes (Schleicher & Schuell, Keene, NH) which are pure nitrocellulose transfer and immobilization membranes with a pore size of 0.45 μm in a BIORAD Mini Trans-Blot Electrophoretic Transfer unit. The unit contained transfer buffer [7.2 gm glycine, Fisher Biotech ; 1.5 gm Trisbase, Sigma 7-9 T-1378; 200 ml methanol; 100 μl PMSF, Sigma P-7626; in 1 litre distilled water]. Conditions for transfer were as follows: run at 100 V, 250 mA for one hour at 4°C in the refrigerator.

Ponceau S Staining of the Blots

To visualize protein bands to facilitate cutting out individual lanes, blots were washed 2×5 minutes in distilled water, followed by incubating in working Ponceau S solution for 5 minutes [one part stock solution (2% Ponceau S in 30% trichloracetic acid and 30% sulfosalicylic acid) and nine parts distilled water]. Blots were rinsed twice with distilled water (2×3 minutes).

Western Blotting

Western blotting was performed to detect antibodies binding to selected food antigens using peroxidase linked secondary antibodies as follows:

- Extracts were electrophoresed, transferred, cut and placed in separate small plastic dishes made from the plastic cover slip from sterile microscope culture slides with a divider glued in the middle to accommodate two blots (1×4.5 cm each).

- A standard SPI extract and pooled control serum was included in each run.
- Washed blot 2×5 minutes in distilled water
- Placed in working Ponceau S stain for 5 minutes, rinsed in distilled water until the lanes were visible
- Marked lanes and molecular marker positions with a soft pencil
- Washed in distilled water until the stain was removed, cut blot into lanes
- washed 3×5 minutes in phosphate buffered saline (PBS)
- Blocked for one hour in blocking buffer (5% skim milk powder in PBS at pH 7.5) with rocking on a Reliable Scientific shaker at speed 3. (Using either gelatin or skim milk powder solution as a blocking buffer did not affect band density).
- Incubated with primary antibody at 1:1 dilution (several different dilutions in a range of 1:1, 1:1.5, 1:2, 1:3, 1:5 up to 1:20 were tested to determine appropriate dilution. Results showed that the best dilution for this purpose was a 1:1 dilution) in 5% skim milk powder in PBS with agitation for one hour.
- Washed 3×5 minutes in PBS.
- Incubated with secondary antibody (DAKO P450-antirat IgG-HRP, rabbit anti-rat IgA-HRP) at ~1:800 and goat anti-human IgG-HRP (Fc specific, Sigma H10007), goat anti-human IgA-HRP (Alpha, Sigma H14007) at ~1:100 dilution in 5% skim milk powder in PBS with agitation for one hour (All the secondary antibodies were affinity purified antibodies and labelled with horse radish peroxidase). Appropriate dilutions were determined experimentally.
- Washed 3×5 minutes in PBS.

Staining of the Blots

Blots were stained using DAB (Sigma D-5637) solution containing 6 mg of 3,3' diaminobenzidine (tetrahydrochloride) in 10 ml of 50 mM Tris-HCl (pH 7.6) and 10 μl 30% H_2O_2 (Fisher Biotech, H323) for 5 minutes, followed by 2 washes of 5 minutes in PBS.

Standardization and Controls for Western Blotting

To reduce the effect of variation from blot to blot in our semi-quantitative analysis, several controls were used: (i) customizing band detection parameters such as sensitivity, minimum density, noise filter, shoulder sensitivity and size scale to standard values to suit the range of values in our images, (ii) there was an SPI control lane in each of our blots in each run which was incubated with the same pooled control serum (pooled from 10 non-diabetes-prone, BBc rats). In the analysis, the immunoreactive S2 band in that lane was used as a control. Each time the absorbance value for the S2 band in the SPI standard lane was compared and adjusted to the standard value by subtracting the difference between standard (0.10) and the standard lane on the test blot. Bands were characterized as absent if their final absorbance reading after adjustment was less than the mean of background absorbance + 2 SD, and present if the absorbance was greater than the mean + 2 SD (i.e., $0.063 + 2\times0.014$), (iii) Western blots for entire experimental groups were carried out in one day to maintain the same conditions across the group, (iv) where possible, solutions were made in large volumes and stored at 4°C and some buffers such as the running and transfer buffers were made in concentrated form (10X volume) to further reduce possible variation.

All the protein extractions were done at once, followed by protein assay and the extracts were aliquoted in appropriate volumes in Eppendorf tubes and stored at -20°C.

By extracting a large amount of material at once, it was possible to use the same extract each time to further reduce variation in the extraction procedure and ensure loading of the same amount of protein per lane for each run. This also meant that extracts were frozen and thawed only once. Similarly, primary antibodies were spun, aliquoted in appropriate amounts for each blot and kept frozen to avoid degradation by repeated freezing and thawing. The secondary antibody used was from the same batch throughout these studies. The diaminobenzidine solution which was used for staining, was also made in a concentrated solution in a large volume, aliquoted and frozen at -20°C.

After proteins were transferred to the nitrocellulose membranes, the gel was stained with Coomasie Blue to confirm degree to which proteins were transferred to the blot. This routinely showed that no protein remained in the gel. Ponceau S staining of the blots was used to verify the presence and location of protein fractions on the membrane. To check for non-specific binding, runs were carried out as usual but omitting the primary serum or applying antiserum from rats fed wheat only. These results showed no non-specific binding of the secondary antibodies and no immunoreactivity against soy fractions in wheat gluten fed rats. Casein or hydrolysed casein extracts were also probed as negative controls and no bands were observed. Blots were photographed immediately following development of the bands to avoid problems with fading or other variations in staining.

Semi-Quantitative Computer-Based Image Analysis

After staining, the blots were photographed using a KODAK DC 40 digital camera and photoenhancer software. The images were saved as colour TIF files and gray TIFs by changing the depth of image to 256 shades of gray. The gray TIF file was then transferred to a UNIX-based PDI image analysis system (PDI Inc., Huntington Station,

NY).

After loading the images, they were quantitatively analyzed using PDI software for analysis of 1D gels, "Quantity One". Bands were characterized as absent if the absorbance was less than the mean of background absorbance + 2 SD, and present if the absorbance was greater than the mean + 2 SD (i.e., 0.063 + 2×0.014). A detailed report of Rf, M.W., Peak OD and relative proportion for each band was obtained. The presence or absence of a band was also verified by eye using the original blot, and this was manually modified when necessary. For example, the band location was entered manually if the software misidentified or missed a faint band. The standard SPI lane was considered as a control lane for all of the analyses, and the quantitative analyses were carried out relative to the S2 band in that lane.

Statistical Analysis of the Data

The results of analysis including peak OD were entered in a spread sheet containing detailed rat or patient information and appropriate protein fraction numbers: S1-S15 for soy, N1-N12 for NIH. The mean±SD for the entered values for each band in each group was calculated, an analysis of variance (ANOVA) was used to measure variability followed by the LSD test to measure significance of differences among means. Fisher's exact test (one or two tailed as appropriate) was used to compare frequency of each band for each food material among different bands with the significance level set as $p \leq 0.05$.

Experimental Design

Experiment 1 Preliminary Identification of Immunogenic NIH and Soy Proteins

Certain soy protein sources have been identified as potential food diabetogens in BB rats and NOD mice. This experiment was designed to investigate which soy protein fractions are immunogenic and possibly diabetogenic. Extracts of several soy protein sources were probed with sera from control (BBc), diabetes-prone (BBdp), diabetes (untreated) BBd-U and insulin-treated diabetic rats, BBd-T. The following soy protein sources were extracted: NIH (containing soymeal), soybean meal, soy protein isolate (SPI), powder and liquid soy-based infant formulas (Isomil, Prosobee) as food antigens, followed by 1D SDS-PAGE and Western blotting. Blots were probed with sera from the aforementioned rat types with ages ranging from 62-138 day from the colonies maintained in the Animal Resources Division of Health Canada. All BB rats were fed a closed formula, cereal-based diet, Purina 5001 from weaning at ~23 d.

Rat anti-soy IgG antibodies (rabbit anti-rat IgG-HRP) were measured in sera from 10 BBc (mean age in days ± SD; 92±24 d), 8 BBdp (85±20 d), 13 BBd-U (82±20 d) and 12 BBd-T (102±23 d) animals. Because insulin receptors appear on activated T cells, an insulin treated group was included to compare the potential effect of treatment on immunoreactivity compared to the untreated diabetic group. Western blotting, image analysis and statistical comparisons were carried out as described above.

Experiment 2 Characterization of the Development of Soy Antibodies with Age in a Cross- Sectional Study of BB Rats

This experiment was designed to investigate if there was an age-related variation in the levels of IgG and IgA antibodies to SPI extract proteins, in BBc and BBdp rats. For this purpose, SPI proteins were extracted, followed by 1D SDS-PAGE and Western

blotting. Purina 5001-fed animals from the HPB colony were selected from BBc and BBdp groups at different ages: 5, 15, 45, 70, and 120 d. Blood from 5 and 15 day old pups was collected following decapitation and samples from entire litters were pooled to obtain sufficient serum. In 45, 70 and 120 d animals, 4-10 individual rats in each age group were anaesthetized and blood samples were collected and processed to obtain serum as described above. Blots were probed with sera from the aforementioned rats at 1 : 1 dilution with blocking buffer. Rabbit anti-rat IgG-HRP and anti-rat IgA-HRP diluted 1 : 800 were used as secondary antibodies. Bands were visualized with DAB, followed by image analysis and statistical comparison.

Experiment 3 A Prospective Study of IgG Antibodies in BBdp Rat Sera that Bind SPI Proteins: Effect of Various Soy Diets

Recently, the American Academy of Pediatrics released a revision of its guideline for infant feeding indicating that infants at risk for developing diabetes should not be fed milk in the first year of life and that soy-based infant formulas should not be fed as a means of prevention. The soy recommendation was based solely on our studies using BB rats fed soybean meal which is a crude and much less refined soy preparation compared to the soy protein isolate commonly used in soy-based infant formulas. Also the soybean protein isolate used to make powdered soy formula is a mildly hydrolysed form of the soy protein isolate used in the liquid soy formula (Burks, Butler, Brooks, Hardin and Connaughton 1988). Therefore, it was important to further characterize the diabetogenicity of the more processed infant formula materials themselves.

It has been shown that food-induced diabetes is a time and dose-dependent interaction (Scott 1996). To expand these findings and consider the effect of age and immunoreactivity against certain diet materials, a prospective study was performed in

BBdp rats fed semipurified, AIN-76A diets containing single protein sources, SPI, hydrolysed SPI, Prosobee powder infant formula, Prosobee liquid infant formula and hydrolyzed casein. The protein intake was equal in all diet groups. At 23 days of age, animals were randomly selected from several litters and assigned to five groups of 17 animals each, placed in individual wire bottom stainless steel cages and weaned onto the above diets.

Body weight was monitored weekly and animals were assessed twice weekly for diabetes beginning at age 55 days. Diabetes was defined as follows: fasting blood glucose ≥ 200 mg/dL, 2+ or greater glucose (Testape, Eli Lilly, Toronto, Ontario) in urine, excessive water consumption, polyuria and weight loss or failure to gain weight. Diabetic rats were killed within 24 hour of confirmed diagnosis by exsanguination while under halothane or isoflurane anaesthesia (3% in O_2). Remaining asymptomatic rats were killed at approximately ~150 days of age.

Two prospective blood samples were taken from the orbital plexus of the eye from each animal while under light anaesthesia in all diet groups at age 45 and 70 days. Another blood sample was taken either at the time of diagnosis of diabetes or at the end of the study at 150 days by exsanguination from the abdominal aorta. Serum was prepared and these samples were used to probe Western blots of SPI extracts for IgG antibodies. Animals were further grouped as asymptomatic or diabetic based on the diabetes outcome in each diet group. This permitted prospective analyses of the appearance of soy antibodies as a function of age, diabetes outcome and overall diabetogenicity of each diet.

Experiment 4 Identification of the Immunogenic Soy Antigens Using Sera from Control and Diabetic Humans

In order to determine if our findings in the BB rat may be applicable to humans,

blood samples from newly diagnosed diabetic children were obtained from Dr.K.A. Faught, Pediatrician at the Children's Hospital of Eastern Ontario (CHEO) in Ottawa and Dr. O. Simell, Professor and Chairman of the Department of Pediatrics, University of Turku, Finland.

The children were aged between 3 and 16 years. Based on the amount of serum available for each patient, Western blots were carried out of protein extracts of known diabetogenic foods such as soy protein isolate and soybean meal. Immunoreactivity was measured using 1:100 dilution of goat anti-human IgG-HRP (Fc specific, Sigma H10007) and goat anti-human IgA (Alpha, Sigma H14007).

The results of greatest interest here were (i) the patterns from Western blots of the sera from untreated human patients, (ii) to what extent these sera identified bands previously inferred to be diabetes-related using BB rat sera, (iii) if there were major differences comparing these samples to sera from non-diabetic humans. For the preliminary analysis (CHEO), a small group of volunteers (not age-matched or HLA-matched) acted as "controls". A large sample set from age, sex and HLA-matched non-diabetic children from Finland was also analyzed.

Experiment 5 Effect of Early Oral Dosing with Soybean Meal on Diabetes Development in BB Rats

It was shown in our laboratory that early oral dosing of BBdp rats, between day 4 and 7 with NIH prevented diabetes in animals subsequently weaned onto the NIH diet. In order to expand this finding, an experiment was carried out to investigate whether early exposure of the gut immune system to soy would have the same protective effect when animals were weaned onto a diabetogenic soy diet.

Neonatal BB rat pups were removed from their dams and hand fed daily from 4 to

7 d of age a mixture of Pregestimil® (a hydrolysed casein-based, non-diabetogenic infant formula, consisting of : 13% hydrolysed casein, 62% carbohydrates which is corn starch and corn syrup solids, modified coconut oil, supplemented with micronutrients, Mead Johnson, Belleville, Ontario) and soybean meal or Pregestimil® alone. The food antigens were given in appropriate dose to deliver ~15 mg protein per feeding. The amount of protein was equivalent to ~1 mg/g body weight, a dose known to induce oral tolerance (Mowat, 1987). Pups usually weighed 12-15 g and were fed through a 1 ml disposable syringe attached to a 21 gauge butterfly with the needle removed. Approximately 0.4 ml of the prepared diet solution was fed to deliver enough protein per feeding. The diet mixture was kept at body temperature during feeding. After feeding, the pups were returned to their dams. Feces and urine soaked bedding were applied to the pups to reduce maternal cannibalism. Littermates were distributed among the treatment groups. In order to tell them apart at weaning, the pups were tattooed on the foot. After four days of hand feeding, the pups remained with the dams until weaning at 23 days. Control, Pregestimil®-treated pups were randomly divided among groups weaned onto a diabetogenic soybean meal diet. Those treated with soybean meal were weaned onto the soybean meal based diet. There were two control groups, a hydrolysed casein-fed group served as negative control and an NIH-fed group served as a positive control group, neither of which was dosed orally.

During the four days of hand feeding, the pups were weighed each day and body weight was monitored weekly beginning at around 30 days. Food consumption was also determined at 75 and 120 days of age. Diabetic rats were killed within 24 hours of confirmed diagnosis and remaining asymptomatic rats were killed at approximately 150 days of age. All animals were killed by exsanguination while under isoflurane anaesthesia

and blood samples were collected from the abdominal aorta.

Western blotting was performed with sera from individual animals in the soy-dosed (dosed orally with soy and weaned onto soy diet) and non-dosed (dosed orally with Pregestimil® and weaned onto soy diet) groups. An extract of the soybean meal fed in this experiment was electrophoresed, blotted, and probed with the aforementioned sera and anti-soy IgG antibodies were measured.

Experiment 6 Pre-absorbing Antibodies from BB Rat Serum with Rat Insulinoma Cells, RINm5F: A Preliminary Study

This experiment was designed to investigate cross reactivity between food antigens and rat insulinoma (RIN) cells which served as a source of β-cell antigens. For this purpose, rat insulinomia (RIN) cells, the RINm5F cell line originally derived from a β-cell tumour induced in NEDH (New England Deaconess Hospital) rats, were supplied from Dr. Ji-Won Yoon, University of Calgary. RIN cells were cultured in 75 cm^2 culture flasks under the following conditions : 37°C, 5% CO_2, 90% relative humidity. Cells were grown in a RIN complete medium, which contained the following in 100 ml: 86 ml RPMI 1640 medium (Gibco-BRL,Burlington, Ontario. # 22400-055), supplemented with 10 ml of 10% fetal bovine serum (FBS, Gibco 39K2356), 1 ml of penicillin-streptomycin stock solution [~100 mg/ml (Gibco, #15140-015], 1 ml of 2 mM L-glutamine (Gibco, #25030-016), 1 ml of 5 mM D-glucose, 1 ml of 1 mM sodium pyruvate.

For pre-absorbing antibodies in BB rat sera, confluent RIN cells were collected. These cells have maximum expression of β-cell antigens when they reach confluency. Each serum sample was pre-absorbed with approximately 1×10^8 cells obtained from a single 75 cm^2 flask by gently detaching the cells with a rubber policeman (to avoid

trypsinization). A small volume of PBS was added to collect cells, followed by pelleting in an Eppendorf centrifuge at full speed for 15 seconds. From this point on, the tubes were placed on ice.

Two different procedures, using unbroken cells and broken cells, were followed for pre-absorbing the antibodies. To obtain unbroken cells, the supernatant was carefully removed with a Pasteur pipette, cells were resuspended in 50 μl of PBS and 950 μl of serum sample, incubated on ice for one hour with gentle shaking by hand every 5-10 minutes, followed by pelleting in the Eppendorf centrifuge for 20 seconds at full speed. Serum was used for Western blotting immediately (without freezing) but stored at 4°C for 30-60 minutes if necessary. To obtain broken cells, RINs were first resuspended in a minimum volume of PBS required for sonication and were sonicated on ice for 30 seconds (microtip limit:3, duty cycle:40%, Cell Disruptor, Heat Systems-Ultrasonics sonicator, model W-375), pelleted in an Eppendorf microfuge for 20 seconds at full speed, then the supernatant was carefully removed with a Pasteur pipette, cells were resuspended in 50 μl of PBS and 950 μl of serum sample, incubated on ice for one hour, pelleted in an Eppendorf for 30 seconds at full speed and serum was used immediately for Western blotting as above.

Western blotting was performed with treated sera as well as untreated sera from the same animal using three different types of rats; BBc, BBdp, BBd; all of whom were fed Purina 5001. The target antigens were from protein extracts of NIH and SPI as described previously. Rat IgG and IgA antibodies bound to the proteins were detected using rabbit anti-rat IgG or IgA labelled with horse radish peroxidase (HRP) and reacted with DAB.

RESULTS

To investigate possible diabetes related soy proteins, proteins extracted from soy materials including NIH, soymeal and SPI were separated using SDS-PAGE gel electrophoresis, followed by Western blotting using sera from BB rats (BBc, BBdp, BBd). Two major issues were addressed, (i) which bands were diabetes-related, that is, differed among control (nondiabetes-prone) and BBdp and BBd rats and (ii) related to pathogenesis, that is changes observed in bands when comparing BBdp (diabetes-prone) to diabetic rats treated with insulin, BBd-T, or untreated, BBd-U. The changes in the frequency (number of rats displaying the band in question/total in group) and intensity (absorbance) of DAB staining were analyzed.

Experiment 1 Preliminary Identification of Immunogenic NIH and Soy Proteins

Various soy protein sources were screened and found to have diabetogenic potential in genetically diabetes-prone BB rats and NOD mice. This diabetogenic activity is present even in highly processed soy materials. Soybean meal, a diabetogenic component of the NIH diet, is a less refined preparation unlike the soy protein isolates (SPI), used in soy-based infant formulas. In previous studies from our laboratory (Scott 1995), diets containing SPI were less diabetogenic in BB rats than soybean meal diets.

Therefore, there were several compelling reasons to characterize diabetes related proteins in more detail: (i) soymeal and associated products were shown to be diabetogenic when fed in semipurified diets to BBdp rats (Hoorfar et al. 1991), (ii) the effects of food processing on soy diabetogenicity must be classified and (iii) infant feeding guidelines were revised to suggest avoiding soy formulas in the diet of susceptible infants (Drash, Kramer, Swanson and Udall 1994).

To identify unique, diabetes-related soy proteins, sera from BB rats of various levels of risk for developing diabetes (BBc, BBdp and BBd) were used as the source of primary antibodies. These animals were fed Purina 5001[1]. NIH (which contains soybean meal), soybean meal, soy protein isolate (SPI) and powder or liquid soy-based infant formulas (Isomil and Prosobee) were extracted with 0.3 mM Tris-HCl buffer, followed by 1D SDS-PAGE gel electrophoresis and Western blotting.

Silver stained bands in SDS-PAGE gels of extracts from NIH and soymeal were labelled sequentially from highest to lowest molecular mass as N1-N12 for NIH diet extracts and S1-S15 for soy food extracts (Fig.1). The protein bands in the soy extract were tentatively identified based on molecular mass, relative amounts and previously published data (Burks et al 1989; Wolf, Peterson and Schaer 1992; Burks, Brooks and Sampson 1988). Some bands in the NIH extract had similar molecular mass to bands in the soy extracts. This is to be expected as soy meal is a major component of NIH.

When various soy products (soymeal, SPI, infant powder and liquid soy-based formulas) were electrophoresed, it was clear that a similar pattern and amount of proteins were present in soymeal and SPI extracts whereas fewer bands and decreased amounts of protein were seen in the infant formulas (Fig. 2); the powdered soy formulas had mainly low molecular mass proteins and peptides and the soy band pattern was not visible. Western blotting of the NIH and SPI protein extracts was performed with individual sera from 10 BBc (mean age$\pm$SD; 92$\pm$24 d), 4-8 BBdp (85$\pm$20), 12-13 diabetic untreated (BBd-U, 82$\pm$20) and 9-12 diabetic, insulin treated (BBd-T, 102$\pm$23) rats in the age range over which diabetes occurs, 62-138 d (Table 1, Figures 3,4). Rat IgG antibodies bound to

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[1] Purina 5001 and NIH rodent feeds have similar compositions but the former is a closed formula diet (% ingredients not given) and the later is an open formula diet (% ingredients known), see Materials and Methods section for details.

the proteins were detected using rabbit anti-rat IgG-HRP antibody reacted with DAB.

Results showed that of the twelve bands evaluated (N1-N12) for NIH, the N6, N7, N8 and N11 bands were significantly different among the four groups of animals based on analysis of the Western blots using ANOVA and observation by eye (Table 1,2, Fig. 3). In blots of the NIH extract, the N6 band was always present but showed less intense DAB staining in Western blots using sera from BBd (0.11 ± 0.04) compared to BBc (0.16 ± 0.06, $p=0.02$) or BBdp rats (0.19 ± 0.05, $p=0.002$). For N7, blots showed less intense bands using sera from BBd rats compared with BBc or BBdp, but this difference was only significant comparing BBd to BBdp rats (0.09 ± 0.06 vs 0.19 ± 0.04, $p=0.004$). The intensity of staining for the N8 band was highest in BBd rats (0.22 ± 0.03) compared with the other three groups and was less frequent in BBc vs BBd-U. The highest absorbance values of all the bands were observed for the N11 band in BBc rats. Both frequency and intensity of binding to this band was higher in BBc rats and virtually absent or very weak in BBdp, BBd-U and BBd-T groups (Table 1, $p<0.0001$). These results suggested that the N6, N7 and N8 bands were diabetes-related, and N11 binding was characteristic of sera from control, BBc rats.

For the SPI extract (Table 1,2, Fig. 4), both the frequency and absorbance values for the S6 band were significantly higher in BBc rats compared with treated or untreated BBd rats. The S6 band in BBdp rats tended to be darker than in BBd rats.

Table 1. Western blot analysis of IgG antibodies in sera from BB rats against NIH and Soy proteins extracts[1,2].

Band	BBc		BBdp		BBd Untreated		BBd Treated	
	Frequency	Absorbance mean±SD	Frequency	Absorbance mean±SD	Frequency	Absorbance mean±SD	Frequency	Absorbance mean±SD
NIH								
N6[3]	10/10	0.16 ± 0.06^{a}	8/8	$0.19\pm0.05^{b,c}$	13/13	$0.11\pm0.04^{a,b}$	12/12	0.12 ± 0.06^{c}
N7	8/10	0.13 ± 0.10	8/8	$0.19\pm0.04^{a,b}$	12/13	0.09 ± 0.06^{a}	10/12	0.10 ± 0.07^{b}
N8[4]	$6/10^{\alpha}$	0.09 ± 0.10^{a}	7/8	0.14 ± 0.07^{b}	$13/13^{\alpha}$	$0.22\pm0.03^{a,b,c}$	9/12	0.11 ± 0.08^{c}
N11	$9/10^{\alpha,\beta,\gamma}$	$0.33\pm0.20^{a,b,c}$	$3/8^{\alpha,\delta,\epsilon}$	0.04 ± 0.07^{a}	$0/13^{\beta,\delta}$	0.00 ± 0.00^{b}	$0/12^{\gamma,\epsilon}$	0.00 ± 0.00^{c}
SPI								
S6	10/10	$0.16\pm0.07^{a,b}$	7/8	0.14 ± 0.06	10/13	0.09 ± 0.09^{a}	10/11	0.08 ± 0.05^{b}
S8	9/10	0.16 ± 0.09^{a}	8/8	0.17 ± 0.07^{b}	$13/13^{\alpha}$	0.12 ± 0.06	$8/11^{\alpha}$	$0.09\pm0.07^{a,b}$
S10	$5/10^{\alpha}$	$0.07\pm0.08^{a,b}$	7/8	$0.15\pm0.06^{a,c}$	$12/13^{\alpha}$	$0.23\pm0.08^{b,c,d}$	7/11	0.10 ± 0.09^{d}
S13	$9/10^{\alpha,\beta,\gamma}$	$0.37\pm0.20^{a,b,c}$	$2/8^{\alpha}$	0.05 ± 0.09^{a}	$1/13^{\beta}$	0.01 ± 0.05^{b}	$0/11^{\gamma}$	0.00 ± 0.00^{c}

[1] Extracts of each NIH or soy material were electrophoresed and submitted to Western blot analyses.
[2] All sera were from animals fed Purina 5001. The sera used for each diet extract were from the same group of animals.
[3] Absorbance values sharing the same letter within rows are significantly different, ANOVA with LSD test, $p \leq 0.05$. For p values for all comparisions see table 2.
[4] Frequency values sharing the same Greek letters within rows are significantly different, Fisher's exact test, $p \leq 0.05$.

Table 1. (Cont'd) Western blot analysis of IgG antibodies in sera from BB rats against NIH and Soy proteins extracts[1,2].

Band	BBc		BBdp		BBd Untreated		BBd Treated	
	Frequency	Absorbance mean±SD	Frequency	Absorbance mean±SD	Frequency	Absorbance mean±SD	Frequency	Absorbance mean±SD
SOYMEAL								
S6[3]	10/10	$0.16\pm0.08^{a,b}$	8/8	$0.18\pm0.07^{c,d}$	10/13	$0.09\pm0.07^{a,c}$	10/11	$0.08\pm0.05^{b,d}$
S8[4]	10/10	0.17 ± 0.09^{a}	8/8	0.17 ± 0.07^{b}	$13/13^{\alpha}$	0.12 ± 0.09	$8/11^{\alpha}$	$0.09\pm0.07^{a,b}$
S10	$6/10^{\alpha}$	0.08 ± 0.07^{a}	6/8	0.11 ± 0.08^{b}	$13/13^{\alpha,\beta}$	$0.24\pm0.03^{a,b,c}$	$7/11^{\beta}$	0.09 ± 0.09^{c}
S13	$9/10^{\alpha,\beta,\gamma}$	$0.35\pm0.17^{a,b,c}$	$1/8^{\alpha}$	0.03 ± 0.08^{a}	$0/13^{\beta}$	0.00 ± 0.00^{b}	$0/11^{\gamma}$	0.00 ± 0.00^{c}
ISOMIL								
S6	$6/10^{\alpha}$	0.09 ± 0.09^{a}	2/7	0.05 ± 0.08	$2/12^{\alpha}$	0.01 ± 0.03^{a}	5/9	0.04 ± 0.04
S8	4/10	0.07 ± 0.09	1/7	0.03 ± 0.09	6/12	0.03 ± 0.04	3/9	0.04 ± 0.06
S10	6/10	0.08 ± 0.07^{a}	4/7	0.09 ± 0.08^{b}	9/12	$0.18\pm0.11^{a,b,c}$	4/9	0.08 ± 0.09^{c}
S13	$7/10^{\alpha,\beta,\gamma}$	$0.24\pm0.20^{a,b,c}$	$1/7^{\alpha}$	0.04 ± 0.09^{a}	$0/12^{\beta}$	0.00 ± 0.00^{b}	$0/9^{\gamma}$	0.00 ± 0.00^{c}
PROSOBEE								
S6	6/10	0.09 ± 0.09^{a}	1/4	0.04 ± 0.09	3/12	0.02 ± 0.05^{a}	4/9	0.04 ± 0.05
S8	3/10	0.05 ± 0.09	0/4	0.00 ± 0.00	5/12	0.04 ± 0.05	4/9	0.05 ± 0.07
S10	5/10	0.06 ± 0.07^{a}	2/4	0.07 ± 0.08^{b}	9/12	$0.18\pm0.11^{a,b,c}$	4/9	0.07 ± 0.08^{c}
S13	$8/10^{\alpha,\beta,\gamma}$	$0.29\pm0.18^{a,b,c}$	$0/4^{\alpha}$	0.00 ± 0.00^{a}	$1/12^{\beta}$	0.02 ± 0.06^{b}	$0/9^{\gamma}$	0.00 ± 0.00^{c}

[1] Extracts of each soy materials were electrophoresed and submitted to Western blot analyses.
[2] All sera were from animals fed Purina 5001. The sera used for each diet extract were from the same group of animals.
[3] Absorbance values sharing the same letter within rows are significantly different, ANOVA with LSD test, $p \leq 0.05$. For p values for all comparisions see table 2.
[4] Frequency values sharing the same Greek letters within rows are significantly different, Fisher's exact test, $p \leq 0.05$.

Table 2. *p* values of mean of absorbances and frequencies for NIH and Soy protein fractions.

NIH

Band	Type	BBc [Frequency]Absorbance	BBdp [Frequency]Absorbance	BBd-U [Frequency]Absorbance	BBd-T [Frequency]Absorbance
N6	BBc	–			
	BBdp	[1.000] 0.354	–		
	BBd-U	[1.000] **0.017**	[1.000] **0.002**	–	
	BBd-T	[1.000] 0.084	[1.000] **0.012**	[1.000] 0.482	–
N7	BBc	–			
	BBdp	[0.294] 0.083	–		
	BBd-U	[0.398] 0.212	[0.619] **0.004**	–	
	BBd-T	[0.632] 0.342	[0.347] **0.009**	[0.469] 0.763	–
N8	BBc	–			
	BBdp	[0.225] 0.216	–		
	BBd-U	**[0.024]** **0.000**	[0.381] **0.022**	–	
	BBd-T	[0.384] 0.604	[0.465] 0.419	[0.096] **0.000**	–
N11	BBc	–			
	BBdp	**[0.032]** **0.000**	–		
	BBd-U	**[0.000]** **0.000**	**[0.042]** 0.345	–	
	BBd-T	**[0.000]** **0.000**	**[0.049]** 0.353	[ns] ns	–

SPI

Band	Type	BBc [Frequency]Absorbance	BBdp [Frequency]Absorbance	BBd-U [Frequency]Absorbance	BBd-T [Frequency]Absorbance
S6	BBc	–			
	BBdp	[0.444] 0.586	–		
	BBd-U	[0.161] **0.026**	[0.502] 0.122	–	
	BBd-T	[0.524] **0.023**	[0.678] 0.105	[0.363] 0.886	–
S8	BBc	–			
	BBdp	[0.556] 0.950	–		
	BBd-U	[0.435] 0.164	[ns] 0.171	–	
	BBd-T	[0.331] **0.032**	[0.170] **0.037**	[0.081] 0.363	–
S10	BBc	–			
	BBdp	[0.120] **0.042**	–		
	BBd-U	**[0.035]** **0.000**	[0.629] **0.029**	–	
	BBd-T	[0.425] 0.352	[0.267] 0.216	[0.112] **0.000**	–
S13	BBc	–			
	BBdp	**[0.009]** **0.000**	–		
	BBd-U	**[0.000]** **0.000**	[0.316] 0.439	–	
	BBd-T	**[0.000]** **0.000**	[0.164] 0.310	[0.542] 0.760	–

Numbers in bold characters indicate $p \leq 0.05$

p values for absorbances were obtained by ANOVA test and for frequencies by Fisher's exact test

Table 2. (Cont'd) p values of mean of absorbances and frequencies for NIH and Soy protein fractions.

SOYMEAL

Band	Type	BBc [Frequency]Absorbance	BBdp [Frequency]Absorbance	BBd-U [Frequency]Absorbance	BBd-T [Frequency]Absorbance
S6	BBc	–			
	BBdp	[ns] 0.550	–		
	BBd-U	[0.162] **0.014**	[0.215] **0.004**	–	
	BBd-T	[0.524] **0.013**	[0.579] **0.004**	[0.363] 0.890	–
S8	BBc	–			
	BBdp	[ns] 0.909	–		
	BBd-U	[ns] 0.210	[ns] 0.196	–	
	BBd-T	[0.124] **0.043**	[0.170] **0.044**	[0.081] 0.362	–
S10	BBc	–			
	BBdp	[0.437] 0.416	–		
	BBd-U	**[0.024]** **0.000**	[0.133] **0.000**	–	
	BBd-T	[0.608] 0.598	[0.494] 0.735	**[0.031]** **0.000**	–
S13	BBc	–			
	BBdp	**[0.002]** **0.000**	–		
	BBd-U	**[0.000]** **0.000**	[0.381] 0.487	–	
	BBd-T	**[0.000]** **0.000**	[0.421] 0.502	[ns] ns	–

ISOMIL

Band	Type	BBc [Frequency]Absorbance	BBdp [Frequency]Absorbance	BBd-U [Frequency]Absorbance	BBd-T [Frequency]Absorbance
S6	BBc	–			
	BBdp	[0.218] 0.132	–		
	BBd-U	**[0.048]** **0.004**	[0.475] 0.253	–	
	BBd-T	[0.605] 0.083	[0.286] 0.907	[0.080] 0.271	–
S8	BBc	–			
	BBdp	[0.278] 0.307	–		
	BBd-U	[0.485] 0.247	[0.144] 0.989	–	
	BBd-T	[0.570] 0.393	[0.392] 0.823	[0.377] 0.810	–
S10	BBc	–			
	BBdp	[0.646] 0.842	–		
	BBd-U	[0.384] **0.015**	[0.378] **0.042**	–	
	BBd-T	[0.414] 0.954	[0.500] 0.805	[0.166] **0.015**	–
S13	BBc	–			
	BBdp	**[0.036]** **0.000**	–		
	BBd-U	**[0.001]** **0.000**	[0.368] 0.502	–	
	BBd-T	**[0.002]** **0.000**	[0.437] 0.526	[ns] ns	–

Numbers in bold characters indicate $p \leq 0.05$

p values for absorbances were obtained by ANOVA test and for frequencies by Fisher's exact test

Table 2. (Cont'd) p values of mean of absorbances and frequencies for NIH and Soy protein fractions.

PROSOBEE									
Band	Type	BBc [Frequency]	BBc Absorbance	BBdp [Frequency]	BBdp Absorbance	BBd-U [Frequency]	BBd-U Absorbance	BBd-T [Frequency]	BBd-T Absorbance
S6	BBc	–							
	BBdp	[0.280]	0.196	–					
	BBd-U	[0.110]	**0.017**	[0.755]	0.605	–			
	BBd-T	[0.414]	0.089	[0.489]	0.968	[0.319]	0.533	–	
S8	BBc	–							
	BBdp	[0.330]	0.195	–					
	BBd-U	[0.454]	0.577	[0.181]	0.355	–			
	BBd-T	[0.430]	0.991	[0.176]	0.199	[0.623]	0.580	–	
S10	BBc	–							
	BBdp	[0.720]	0.910	–					
	BBd-U	[0.221]	**0.006**	[0.365]	**0.047**	–			
	BBd-T	[0.586]	0.948	[0.657]	0.951	[0.166]	**0.009**	–	
S13	BBc	–							
	BBdp	**[0.015]**	**0.000**	–					
	BBd-U	**[0.001]**	**0.000**	[0.750]	0.783	–			
	BBd-T	**[0.001]**	**0.000**	[ns]	ns	[0.571]	0.719	–	

Numbers in bold characters indicate $p \leq 0.05$

p values for absorbances were obtained by ANOVA test and for frequencies by Fisher's exact test

Absorbance values for the S8 band were similar in both BBc and BBdp and both these means were significantly higher compared with the BBd-T group. The absorbance values for the S10 band were highest in BBd-U rats compared with BBc, BBdp and BBd-T. Both the frequency and absorbance values for the S13 band were very high in BBc rats and were absent or very low in BBdp, BBd-U and BBd-T groups (Table 1, $p < 0.0001$). A similar pattern was seen in all other soy materials (soymeal, Isomil and Prosobee liquid infant formulas), except for some minor differences in the immunoreactivity against the S6 and S8 bands in Isomil and Prosobee liquid soy-based infant formulas (Table 1). However, absorbance values for the S6 band still showed a significant difference comparing BBc rats with BBd-U. The S8 band did not show any

significant difference among the groups. In the other soy materials, the electrophoretic pattern was essentially the same for all the protein sources except the soy-based powder infant formulas, in which only low molecular mass proteins were seen. Western blotting was not carried out on these extracts.

Overall, the pattern indicated lower absorbance values for the S6 band in all BBd blots compared with BBc and BBdp regardless of soy protein source. In general, absorbance values of the S8 band were low in BBd-T rats compared with BBc and BBdp for proteins extracted from SPI and soymeal but not in the case of proteins extracted from the two soy liquid formulas. Absorbance values for the S10 band were highest in BBd-U rats and were significantly lower in the three remaining groups of animals (BBc, BBdp, BBd-T). Both the frequency and absorbance values of the S13 band were highest in BBc rats and significantly lower or absent in BBdp, BBd-U and BBd-T animals.

Overall, the N6, N7 and N8 bands in the NIH extract and the S6, S8 and S10 bands in the soy meal extract were significantly different between diabetic (BBd-U) and diabetes-prone rats. Several differences were significant for these bands among control rats and the other three groups, suggesting these bands were diabetes-related. The N11 band in NIH and the S13 band in soy protein extracts were significantly more frequent and more intense using control sera compared with sera from the other three groups of rats suggesting this band was specific to control, BBc rats. It can be inferred (but not proven) from the pattern of reactivity as well as the molecular mass of the reactive bands that the N6 band in NIH resembles the S6 band in soy, the N7 band resembles the S8 band, the N8 band resembles the S10 band and the N11 band in NIH resembles the S13 band in soy materials.

Experiment 2 Characterization of the Development of Soy Antibodies With Age in a Cross- Sectional Study of BB Rats

To study in more detail the immunoreactivity of certain diabetes-related soy protein fractions identified in Experiment 1, the effect of age on frequency of IgG and IgA soy antibodies in sera from BB rats was measured. Two groups of Purina-fed BBc and BBdp rats at 5, 15, 45, 70 and 120 d were obtained from the HPB colony. Serum IgG and IgA immunoreactivity was measured against SPI protein extracts. Sera from entire litters of 5 and 15 day old pups were pooled while individual serum samples were obtained from the 45, 70 and 120 d old animals.

The frequency results indicated that the prevalence of IgG antibodies against the S6 band in SPI in both BBc and BBdp rats increased with age, probably beginning some time between 15 and 45 d; there were no antibodies detected against soy proteins at 5 and 15 d (Fig. 5). Immunoreactivity against this band was more frequent at 45 d in the BBc group compared with BBdp rats. IgG antibodies against the S8 band for both BBc and BBdp groups increased with age and peaked at 45 d. The frequency of animals with an S10 band for both BBc and BBdp groups increased with age, and was higher and almost statistically significantly different at 45 d and 70 d comparing BBdp and BBc rats ($p=0.06$, $p=0.08$ respectively). The S13 band was essentially only seen in BBc rats at 70 d or later.

IgA antibodies against the S6 band in both BBc and BBdp rats occurred most frequently in older rats aged 120 d (Fig. 6) and were less prevalent than IgG antibodies. IgA antibodies against the S8 band in both BBc and BBdp groups peaked at 45 d and then declined sharply. At 120 d, they were absent in BBc rats but remained in 20-25% of BBdp rats. There were no IgA antibodies detected against the S10 and S13 bands at any

age in either BBc or BBdp rats.

Overall, for both control and diabetes-prone rats, there were more bands and these were darker using anti-rat IgG compared with anti-rat IgA as the secondary antibody. The IgG results showed that the S6 band was more frequent in control rats compared with BBdp animals and the S8 band was seen with equal frequency in both BBdp and BBc animals. The S10 band was more frequent in the pre-diabetic period in either 45 d or 70 d old BBdp rats compared with BBc animals and the S13 band was again seen mainly in BBc animals and was virtually absent in BBdp rats.

Experiment 3 A Prospective Study of IgG Antibodies in BBdp Rat Sera that Bind SPI Proteins: Effect of Various Soy Diets

Recently, the American Academy of Pediatrics revised its guidelines for infant feeding indicating that infants at risk for developing diabetes should not be fed cow milk in the first year of life and that soy-based infant formulas should not be fed as a means of preventing juvenile diabetes. The soy recommendation was based solely on data from our laboratory using BB rats fed soybean meal. However, soybean meal is a crude soy preparation compared with the soy protein isolate (SPI) commonly used in soy-based infant formulas. The previous Experiment 2, was a cross sectional study in which BB rats were randomly chosen at various ages from the colony; all animals were fed a cereal-based (soy-containing), Purina 5001 diet. Therefore, it was important to (i) evaluate the diabetogenicity of SPI itself and related SPI-based infant formulas, (ii) obtain prospective blood samples to determine age-related differences in soy antibodies in individual rats that became diabetic compared with those remaining asymptomatic at the end of the experiment, (iii) to have more detailed information on the IgG antibody results from Experiment 2.

Therefore, a prospective study was carried out in 5 groups of BBdp rats fed isocaloric and isonitrogenous semipurified, AIN-76A diets (Fig. 7). Diets containing suspected or known diabetogenic protein sources: SPI, Prosobee liquid or powder soy-based infant formula or potential non-diabetogenic protein sources: hydrolysed SPI and hydrolysed casein, were fed from 23 to 149 d of age. Blood samples were collected at 45, 70 and 149 d or at diagnosis of diabetes. The final diabetes incidence from lowest to highest frequency was: hydrolysed casein (8%; negative control), Prosobee liquid soy-based infant formula, 19%, hydrolysed soy protein isolate, 29%, Prosobee powder soy-based infant formula, 35%, soy protein isolate, 41%. Once the diabetes outcome was known for all animals up to age 149 d, at the end of the experiment, animals were designated as asymptomatic or diabetic. Based on this information, samples from animals at 45 d and 70 d could be classified as asymptomatic or pre-diabetic.[2] The intensity (darkness of the band ≈ absorbance above background) and presence or absence of the band, representing IgG antibodies bound to proteins extracted from SPI, were measured in prospective blood samples from each animal. Comparisons were made for three diet groups, SPI, Prosobee powder and liquid soy-based infant formulas as follows: (i) among asymptomatic rats at each age (45, 70, 149 d), (ii) within pre-diabetic or diabetic animals at the same ages, (iii) among asymptomatic vs pre-diabetic animals at 45 and 70 d for each individual diet group. The major findings were as follows:

SPI diet

Frequency of reactivity against the S6 and S8 bands decreased with age. The intensity of binding showed a similar pattern and this difference was significant for the S8

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[2] Note: It is important to distinguish the term diabetes-prone from pre-diabetic. At the start of the experiment, all the animals are considered to be diabetes-prone, that is they possess the susceptibility to develop diabetes. In order to designate an animal as "pre-diabetic", must work backward from having diagnosed the animal as diabetic.

band when comparing 45 d to 149 d samples. For asymptomatic animals in the SPI-fed group the frequency of immunoreactivity (Table 3, Figure 8) against the S10 and S13 bands was 20-30% and this did not change with age.

Comparing frequency of binding among pre-diabetic or diabetic rats (right column, Fig. 8) at different ages, the S6 band was less prevalent at 45 d (14%), increased to 57% at 70 d and then fell to 28% at ~96 d. The S8 band was present in a majority of rats (71-100%) regardless of age. In the case of the S10 band, immunoreactivity remained high and was in the range of 71-86% at all ages. There was no immunoreactivity against the S13 band at any age.

Comparing bands from blots using sera from asymptomatic vs pre-diabetic rats, at 45 days, immunoreactivity against the S6 band was markedly less frequent (9/10 vs 1/7, $p=0.004$) and less intense (0.19 ± 0.13 vs 0.02 ± 0.04, $p=0.004$) in the pre-diabetic rats (Table 3, Fig. 8). The S8 band was more frequent and the bands were more intense at 70 d in the pre-diabetic or diabetic rats compared with asymptomatic animals but this was not statistically significant. Immunoreactivity against the S10 band was more frequent and more intense in pre-diabetic or diabetic rats compared with asymptomatic animals. These differences were significant for frequency at all ages and for intensity at 70 d. Immunoreactivity against the S13 band was present in 20-30% of asymptomatic rats, and was absent in pre-diabetic or diabetic rats.

Prosobee powder diet

In sera from animals fed Prosobee powder soy-based infant formula (Table 3, Fig. 9), in general, the frequency and intensity of the immunoreactivity against the S6 band decreased with age in asymptomatic and pre-diabetic sera. The decrease in intensity was significant in both groups comparing 45 d sera to 70 d sera. Both the frequency and

Table 3. Western blot analysis of IgG antibodies in sera from BBdp rats fed various soy diets [§]

SPI	Asymptomatic Rats			Pre-diabetic or Diabetic Rats†		
Band	Age	Frequency	Absorbance mean±SD	Age	Frequency	Absorbance mean±SD
S6	45 d	9/10$^{\beta}$	0.19±0.13^{b}	45 d	1/7$^{\beta}$	0.02±0.04^{a,b}
	70 d	6/10	0.14±0.13	70 d	4/7	0.16±0.11^{a}
	149 d	5/10	0.12±0.12	96 d	2/7	0.06±0.11
S8	45 d	9/10	0.16±0.03^{a}	45 d	6/7	0.13±0.07
	70 d	6/10	0.13±0.11	70 d	7/7	0.19±0.05
	149 d	3/10	0.07±0.11^{a}	96 d	5/7	0.14±0.09
S10	45 d	3/10$^{\alpha}$	0.04±0.07	45 d	6/7$^{\alpha}$	0.11±0.05
	70 d	2/10$^{\alpha'}$	0.04±0.08^{b}	70 d	5/7$^{\alpha'}$	0.17±0.13^{b}
	149 d	2/10$^{\alpha''}$	0.05±0.11	96 d	5/7$^{\alpha''}$	0.13±0.09
S13	45 d	3/10	0.05±0.08	45 d	0/7	0.00±0.00
	70 d	3/10	0.06±0.10	70 d	0/7	0.00±0.00
	149 d	2/10	0.05±0.10	96 d	0/7	0.00±0.00

INF. PWD.	Asymptomatic Rats			Pre-diabetic or Diabetic Rats†		
Band	Age	Frequency	Absorbance mean±SD	Age	Frequency	Absorbance mean±SD
S6	45 d	6/11	0.12±0.12^{a}	45 d	3/6	0.12±0.13$^{a'}$
	70 d	2/11	0.03±0.06^{a}	70 d	0/6	0.00±0.00$^{a'}$
	149 d	4/11	0.06±0.08	96 d	1/6	0.03±0.08
S8	45 d	3/11	0.06±0.11^{b}	45 d	4/6	0.17±0.13$^{b,c,c'}$
	70 d	1/11	0.01±0.04	70 d	0/6	0.00±0.00^{c}
	149 d	1/11	0.15±0.05	96 d	0/6	0.00±0.00$^{c'}$
S10	45 d	2/11	0.04±0.09	45 d	1/6	0.04±0.09
	70 d	0/11$^{\alpha}$	0.00±0.00^{a,b}	70 d	3/6$^{\alpha}$	0.12±0.13^{b}
	149 d	4/11	0.05±0.08^{a}	96 d	1/6	0.04±0.09
S13	45 d	3/11	0.07±0.12$^{a,a'}$	45 d	2/6	0.08±0.13$^{a'',a'''}$
	70 d	0/11	0.00±0.00^{a}	70 d	0/6	0.00±0.00$^{a''}$
	149 d	0/11	0.00±0.00$^{a'}$	96 d	0/6	0.00±0.00$^{a'''}$

§ Proteins extracted from SPI, electrophoresed, and transferred to nitrocellulose paper were used as the substrate to evaluate binding of antibodies in rat sera from all diet groups.

a, α: $0.01 < p \leq 0.05$, b, β: $0.001 < p \leq 0.01$, c: $0.0001 < p \leq 0.001$

† Animals aged 45 and 70 d were pre-diabetic, for diabetic rats, mean age at onset was 96±11(SD)

Table 3. (Cont'd) Western blot analysis of IgG antibodies in sera from BBdp rats fed various soy diets §

INF. LIQ.	Asymptomatic Rats			Pre-diabetic or Diabetic Rats		
Band	Age	Frequency	Absorbance mean±SD	Age	Frequency	Absorbance mean±SD
	45 d	2/13	0.02±0.06	45 d	1/3	0.05±0.08
S6	70 d	0/13	0.00±0.00[a]	70 d	0/3	0.00±0.00
	149 d	4/13	0.06±0.09[a]	96 d†	0/3	0.00±0.00
	45 d	7/13	0.10±0.10	45 d	0/3	0.00±0.00
S8	70 d	9/13	0.14±0.10[a]	70 d	0/3	0.00±0.00[a]
	149 d	8/13	0.11±0.09	96 d†	2/3	0.15±0.13
	45 d	1/13	0.02±0.06	45 d	1/3	0.04±0.07
S10	70 d	5/13	0.08±0.11	70 d	1/3	0.05±0.08
	149 d	5/13	0.07±0.09	96 d†	1/3	0.07±0.12
	45 d	1/13	0.02±0.06	45 d	0/3	0.00±0.00
S13	70 d	1/13	0.02±0.07	70 d	0/3	0.00±0.00
	149 d	3/13	0.05±0.09	96 d†	0/3	0.00±0.00

§ Proteins extracted from SPI, electrophoresed, and transferred to nitrocellulose paper were used as the substrate to evaluate binding of antibodies in rat sera from all diet groups.

a, α: $0.01 < p \leq 0.05$, b, β: $0.001 < p \leq 0.01$, c: $0.0001 < p \leq 0.001$

† Animals aged 45 and 70 d were pre-diabetic, for diabetic rats mean age at onset was 96±11 (SD)

intensity of the S8 band were highest in pre-diabetic animals at 45 d. The intensity of immunoreactivity against this band was higher in pre-diabetic rats (0.17±0.13) compared with the asymptomatic group (0.06±0.11, p=0.009). Both the frequency (3/6 vs 0/11, p=0.02) and intensity (0.12±0.13 vs 0.00±0.00, p=0.007) of the immunoreactivity against the S10 band were higher at 70 d in pre-diabetic animals compared with asymptomatic animals. The immunoreactivity against the S13 band was only seen at 45 d in both asymptomatic and pre-diabetic animals.

Prosobee liquid diet

In sera from animals fed Prosobee liquid soy-based infant formula (Table 3, Fig.

10) both the frequency and intensity of the immunoreactivity against the S6 band were the highest at 149 d in asymptomatic rats and the increase in the intensity of binding was significant comparing 70 d to 149 d animals. The frequency and intensity of the immunoreactivity against the S8 band remained high in asymptomatic rats at all ages, while it was relatively high only at ~96 d in diabetic animals and was completely absent in 45 d and 70 d pre-diabetic rats. This difference was significant for intensity of binding at 70 d in pre-diabetic animals compared with asymptomatic rats. The S10 band was seen in one out of three pre-diabetic or diabetic animals at all ages. There was no immunoreactivity against the S13 band in pre-diabetic or diabetic animals at any age. Because of the small number of animals that developed diabetes on this diet, comparisons among pre-diabetic and diabetic rats are questionable and therefore, have not been emphasized.

Overall, comparing patterns from animals fed SPI and Prosobee powder soy-based infant formula diets indicated that in the pre-diabetic period, either 45 d or 70 d or at diagnosis of diabetes, the S10 band was present in most animals in the SPI-fed group. The S10 band was also present in 50% of animals fed Prosobee powder soy-based infant formula in the late pre-diabetic period at 70 d. There was a lack of reactivity against the S6 band at 45 d in the SPI-fed group but the same pattern was not seen in the Prosobee powder fed-group. This discrepancy may be related to the use of SPI as the substrate for Western blots in both groups.

Effect of Diets With Different Diabetogenicity on Frequency of the S6, S8, S10, S13 Bands

To see if diets with different diabetes-inducing potential could affect frequency of appearance of diabetes related bands (S6, S8, S10, S13), simple correlations between band

frequency and diabetes incidence were determined (Fig. 11-14). Immunoreactivity against the S6 band correlated positively with diabetes incidence at all ages in asymptomatic rats and was significant at 45 d (Fig. 11, r=0.95, p=0.05). S8 immunoreactivity also increased but only in pre-diabetic rats (45 d, 70 d) and was significant at 45 d (Fig. 12, r=0.97, p=0.03). The immunoreactivity against the S10 band increased as diabetes incidence increased among diet groups during the pre-diabetic or diabetic period and this nearly reached statistical significance at 70 d in pre-diabetic rats (Fig. 13, r=0.92, p=0.07). The S13 band frequency correlated positively with diabetes incidence in asymptomatic animals at 45 d (Fig. 14, r=0.98, p=0.02) but in general, the S13 band was infrequent in all BBdp rats regardless of age or diabetes status.

Experiment 4 Identification of the Immunogenic Soy Antigens Using Sera from Control and Diabetic Humans

In order to expand our preliminary results with BB rats, two sets of serum samples from newly diagnosed diabetic children were obtained from Dr. K. A. Faught of the Children's Hospital of Eastern Ontario (CHEO; Table 4, n=7, Age, 8±4 y) and Dr. O. Simell of the Department of Pediatrics, Children's Hospital, Turku, Finland (Table 5, n=30, Age, 9±4 y). There were two control samples: (i) for the CHEO study, five non-diabetic volunteers (Table 4, n=5, Age, 35±10 y) who were not age-matched, sex-matched or HLA-matched with our diabetic subjects who donated blood samples and (ii) control samples for the Finnish study were from a larger, age-matched group of Finnish children (Table 6, n=37, Age, 10±6 y).

A Preliminary Study of Diabetic Sera From Patients at CHEO

The CHEO serum samples along with control samples were applied to protein blots of soy bean meal and SPI using conditions similar to those derived for BB rats. Binding

of antibodies was visualized by applying goat anti-human IgG-HRP antibodies followed by staining with DAB.

There were no significant differences for S6 and S8 bands (Table 7, Figures 15, 16). IgG antibodies against the S10 band in soybean meal were more frequent in diabetic children compared to controls (86% vs 20%, $p=0.05$) and more intense (0.18 ± 0.12 vs 0.05 ± 0.10, $p=0.07$). There was a trend showing more reactivity against the S13 band in controls compared with diabetic patients. A similar pattern was seen when SPI antigens were used as target antigens. Overall, these results suggested the S10 and S13 bands were important markers of the presence of diabetes (S10) or absence of diabetes (S13).

Finland study

The Finnish serum samples along with control samples were applied to blots of SPI proteins. IgG antibodies against the S6 band in SPI (Table 8, Figures 17, 18) were more frequent in controls compared with diabetic patients (33/37 vs 16/30, $p=0.001$). The reactivity against the S8 band was more intense in controls compared with diabetic patients (0.19 ± 0.16 vs 0.11 ± 0.10, $p=0.03$). Antibodies against the S10 band were more frequent (23/30, 77% vs 20/37, 54%, $p=0.05$) and more intense (0.20 ± 0.14 vs 0.09 ± 0.08, $p=0.0006$) in diabetic patients. IgG antibodies against the S13 band were less frequent (9/30, 30% vs 25/37, 68%, $p=0.002$) and less intense in diabetic patients compared with control patients.

Table 4. Description of newly diagnosed Type I diabetic children from CHEO and Controls

Patient No.	Sex	Age (y)	Recent infection	Family History	Date of blood sample	Fasting blood sugar (mmol/L)
Diabetic Children						
1	M	6†	No	No	09/94	31.0
2	M	9	No	No	07/94	30.2
3	F	14	No	Yes	07/94	34.6
4	M	8	Yes	Yes	07/94	26.4
5	M	8	No	Yes, Type I	06/94	40.0
6	F	2	Yes	No	12/94	45.0
7	F	5	Yes	Yes, Type I	12/94	26.4
8	M	10	Yes	No	01/95	41.0
9	F	6	Yes	No	08/94	22.7
10	M	13	Yes	No	02/95	23.4
Controls						
1	M	49	No	Yes, Type II	03/96	-
2	F	28	No	Yes, Type I	03/96	-
3	F	24	No	No	03/96	-
4	F	41	No	Yes, Type I	03/96	-
5	M	34	No	No	03/96	-

†Age at onset of diabetic symptoms

Table 5. Description of newly diagnosed Type I diabetic children from Finland.

Patient No.	Sex	Age at onset of symptoms (year)	Recent infection	Family History	Date of blood sample	BG before insulin treatment (mmol/L)	BG after insulin treatment (mmol/L)
1	F	7	No	No	29/05/96	5.7	11.5
2	M	12	Yes	Yes, Type I	29/05/96	9.1	6.2
3	M	9	Yes	No	29/05/96	27.4	10.9
4	M	13	No	Yes, Type II	29/05/96	22.5	12.8
5	M	10	Yes	No	29/05/96	16.1	–
6	F	7	Yes	No	29/05/96	25.5	8.2
7	M	5	No	No	29/05/96	27.4	23.8
8	M	8	No	Yes	29/05/96	20.7	–
9	F	3	Yes	No	29/05/96	27.6	11.4
10	M	2	No	No	29/05/96	41.7	23.3
11	M	3	No	Yes, Type I	29/05/96	12.6	9.7
12	F	3	Yes	Yes, Type II	29/05/96	7.6	7.8
13	M	7	Yes	Yes, Type II	29/05/96	40.3	9.5
14	M	8	Yes	No	29/05/96	40.1	6.4
15	M	15	Yes	Yes, Type I	29/05/96	22.7	12.1
16	M	10	Yes	No	29/05/96	18.7	7.8
17	M	11	Yes	No	29/05/96	17.4	9.8
18	M	10	Yes	No	29/05/96	25.0	7.9
19	F	4	No	Yes, Type II	29/05/96	26.5	10.4
20	F	3	Yes	Yes, Type II	29/05/96	21.3	13.0
21	F	6	No	No	29/05/96	20.0	9.6
22	F	13	No	No	29/05/96	19.7	4.1
23	F	7	No	No	29/05/96	54.5	19.7
24	F	2	Yes	Yes, Type I	29/05/96	18.4	2.2
25	F	5	No	Yes, Type I	29/05/96	29.9	8.4
26	M	3	No	Yes, Type I	29/05/96	14.5	8.8
27	M	16	No	Yes, Type II	29/05/96	31.8	14.3
28	F	7	Yes	No	29/05/96	11.6	9.4
29	M	8	No	Yes	29/05/96	27.2	15.2
30	M	3	No	No	29/05/96	41.5	11.8

Table 6. Information on control children from Finland.

Patient No.	Age at blood sampling (year)	Sex	Recent infection	Family History	Date of blood sample
1	7	M	No	No	04/09/96
2	9	M	No	No	04/09/96
3	13	M	No	No	04/09/96
4	5	M	No	No	04/09/96
5	17	M	No	No	05/09/96
6	15	F	No	No	05/09/96
7	8	M	No	No	04/09/96
8	13	M	No	No	09/09/96
9	8	M	No	No	10/09/96
10	4	F	No	No	10/09/96
11	18	M	No	No	10/09/96
12	14	F	No	No	11/09/96
13	5	M	No	No	11/09/96
14	12	F	No	No	11/09/96
15	8	F	No	No	13/09/96
16	11	M	No	No	13/09/96
17	13	F	No	No	12/09/96
18	8	M	No	No	19/09/96
19	8	F	No	No	20/09/96
20	5	F	No	No	21/09/96
21	16	F	No	No	22/09/96
22	10	F	No	No	22/09/96
23	24	M	No	No	23/09/96
24	26	M	No	No	24/09/96
25	15	F	No	No	25/09/96
26	11	F	No	No	26/09/96
27	15	M	No	No	27/09/96
28	5	M	No	No	29/09/96
29	5	F	No	No	29/09/96
30	2	F	No	No	29/09/96
31	7	M	No	No	11/09/96
32	15	F	No	No	10/09/96
33	6	M	No	No	16/09/96
34	6	M	No	No	17/09/96
35	6	F	No	No	24/09/96
36	5	M	No	No	17/09/96
37	6	M	No	No	24/09/96

Table 7. Western blot analysis of IgG antibodies against soy protein extracts in sera from control and newly diagnosed (untreated) diabetic patients (Ottawa study)

	IgG			
	Control		Diabetic Patients	
Band	Frequency	Absorbance	Frequency	Absorbance
SOY MEAL				
S6	5/5	0.17±0.06	5/7	0.14±0.13
S8	4/5	0.12±0.08	6/7	0.19±0.13
S10	1/5*	0.05±0.10	6/7*	0.18±0.12
S13	3/5	0.14±0.14	1/7	0.04±0.10
SPI				
S6	4/5	0.13±0.09	5/7	0.19±0.09
S8	3/5	0.13±0.12	4/7	0.14±0.16
S10	1/5*	0.05±0.11	6/7*	0.18±0.12
S13	3/5	0.13±0.13	2/7	0.07±0.11

* significant at $p=0.05$

Table 8. Western blot analysis of IgG and IgA antibodies in sera from newly diagnosed Type I diabetic children against SPI protein extract (Finland study)

	Control		Diabetic Patients		*p* value	
Band	Frequency	Absorbance	Frequency	Absorbance	Frequency	Absorbance
IgG						
S6	33/37*	0.15±0.08	16/30*	0.14±0.15	0.001	
S8	25/37	0.11±0.10*	19/30	0.19±0.16*	0.46	0.03
S10	20/37*	0.09±0.08*	23/30*	0.20±0.14*	0.05	0.0006
S13	25/37*	0.12±0.10	9/30*	0.09±0.14	0.002	
IgA						
S6	23/33*	0.11±0.09*	2/14*	0.02±0.05*	0.0006	0.001
S8	18/33*	0.08±0.09*	2/14*	0.02±0.06*	0.01	0.02
S10	20/33*	0.09±0.09*	14/14*	0.16±0.02*	0.004	0.01
S13	9/33*	0.03±0.05	0/15*	0.00±0.00	0.005	

* significant at $p \leq 0.05$

IgA immunoreactivity against the S6 band in SPI was more frequent (23/33, 70% vs 2/14, 14%, p=0.000) and more intense (0.11±0.09 vs 0.02±0.05, p=0.001) in controls. S8 bands were more frequent and more intense in control patients compared with diabetic patients. The IgA immunoreactivity against the S10 band was more frequent (14/14 vs 20/33, p=0.004) and more intense (0.16±0.02 vs 0.09±0.09, p=0.01) in diabetic patients. Immunoreactivity against the S13 band was less frequent in diabetic patients vs controls (0/15 vs 9/33, p=0.005).

Overall, in both the CHEO and the Finland study, the frequency of immunoreactivity against the S6 band was lower in diabetic patients compared with the control group. Also, both the frequency and intensity against the S10 band were higher in diabetic patients while the immunoreactivity against S13 was lower. Therefore, the results obtained using human patient and control sera were remarkably similar to those obtained using sera from BB rats.

The HLA haplotype data of newly diagnosed diabetic children were supplied. HLA-haplotypes in the Finnish population known to be significantly associated with either susceptibility to or protection against IDDM were considered (Table 9). Haplotype A2/B56/DR4 has been identified as one which is highly associated with diabetes in Finnish patients and five of the patients had that haplotype. Among other diabetes-related markers, A24 and B8, eight (27.6%) and thirteen (44.8%) of the patients had these alleles respectively. Sixteen (55%) patients had A2, sixteen (55%) had DR3, seventeen (58.6%) had DR4, eighteen (62%) had DQ2, fifteen (51.7%) had DQ3 and twelve (41.4%) had DQ1 haplotype while only one (3.4%) had DR7, three (10.3%) had DR8, two (6.9%) had DR9, one (3.4%) had DR2 and four (13.8%) had DR13 as negatively associated haplotypes. Overall, twenty three (79.3%) of the patients had a combination of

DR4/DQ3 or DQ2 and sixteen (55.2%) had the DR3/DQ2 highly susceptible haplotypes. However, there were no apparent relation among haplotypes and reactivity against the soy diabetes-related bands.

Table 9. HLA-haplotype data of Finnish newly diagnosed type 1 diabetic children.

Patient No.	HLA-Haplotype																	
	A2	A24	B8	B15	B56	DR1	DR2	DR3	DR4	DR7	DR8	DR9	DR13	DQ1	DQ2	DQ3	DQ4	DQ8
1						X			X					X				X
2	X								X				X	X		X		
3			X			X		X						X	X			
4	X		X		X			X	X						X	X		
5			X					X	X						X	X		
6	X	X							X							X		
7	X				X			X	X						X	X		
8	X			X					X	X					X			X
9	X					X			X					X		X		
10	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
11		X	X					X	X						X			X
12	X	X			X							X	X	X		X		
13	X			X					X			X				X		
14		X						X					X	X	X			
15			X			X		X						X	X			
16			X					X	X						X	X		
17	X		X			X		X						X	X			
18		X				X	X							X				
19			X					X							X			
20		X						X			X				X		X	
21	X		X		X			X	X						X	X		
22	X					X					X			X				
23	X	X							X							X		
24			X			X		X						X	X			
25	X				X	X			X					X		X		
26	X								X		X					X	X	
27	X		X					X	X						X	X		
28	X				X			X	X						X	X		
29			X					X							X			
30		X	X										X		X			
Total	16	8	13	2	5	9	1	16	17	1	3	2	4	12	18	15	2	3

Experiment 5 Effect of Early Oral Dosing With Soybean Meal on Diabetes Development in BB Rats

In order to examine the effect of oral exposure in early infancy to antigens associated with diabetogenesis in the BB rat, animals were dosed orally in early infancy from day 4-7 d with soybean meal suspended in Pregestimil® vehicle[3]. After oral dosing, animals were weaned at approximately 23 d to a soybean meal diet.

Oral exposure in early infancy to a diabetogenic soybean meal delayed disease progression, and mean age of onset of diabetes was increased (72±19 vs 101±25 d; p=0.001; n=15/group, Fig. 19). By the end of the experiment, at 150 d, in the group which was not dosed orally with soy, 7/15 (47 %) animals developed diabetes whereas in the soy dosed group 5/15 (33 %) developed the disease and the rate of onset was decreased.

IgG antibodies against soybean meal proteins from an extract of the soybean meal diet used in this experiment were measured in blood samples from individual rats. Results were analysed in two ways: (i) without considering if an animal became diabetic by the end of the study and (ii) dividing them into two groups of asymptomatic and diabetic rats based on diagnosis of diabetes at the end of the study. Results in the first analysis (Table 10) showed that there was immunoreactivity against the S6, S8, S10 and S13 bands in both groups. In the soy-dosed group, immunoreactivity against the S6 was more frequent (10/15 vs 4/15, p=0.03) and more intense (0.16±0.13 vs 0.07±0.13, p=0.07). IgG antibodies against the S10 band were less frequent in the soy-dosed group compared with the animals which were not dosed orally with soy. Reactivity against the S13 band was seen only in 7-13 % of the animals in both groups.

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[3]Pregestimil® is a non-diabetogenic hydrolysed casein-based infant formula.

Table 10. Comparison of differences in immunoreactivity of soymeal fractions using sera from BBdp rats dosed orally with soymeal

Band	Diet	Oral Tx Day 4-7	n	Freq.	Absorbance	*p* value Freq.	*p* value Absorbance
S6	Soymeal	Soymeal in Pregestimil	15	10/15	0.16±0.13	0.03	0.07
	Soymeal	Pregestimil	15	4/15	0.07±0.13		
S8	Soymeal	Soymeal in Pregestimil	15	9/15	0.14±0.12	0.21	0.24
	Soymeal	Pregestimil	15	12/15	0.19±0.11		
S10	Soymeal	Soymeal in Pregestimil	15	4/15	0.06±0.12	0.22	0.26
	Soymeal	Pregestimil	15	7/15	0.12±0.13		
S13	Soymeal	Soymeal in Pregestimil	15	2/15	0.03±0.09	0.50	0.63
	Soymeal	Pregestimil	15	1/15	0.02±0.07		

The second analysis in which rats were grouped as asymptomatic or diabetic (Table 11) highlighted additional significant differences. In asymptomatic rats, IgG antibodies against the S6 band were more frequent (7/10 vs 1/8, p=0.02) and more intense (0.18±0.13 vs 0.03±0.07, p=0.02) in the soy-dosed group. In orally dosed diabetic rats, antibodies against the S8 band were less intense (0.08±0.11 vs 0.23±0.11, p=0.03) and the S10 band was less frequent (1/5 vs 6/7, p=0.05) and less intense (0.03±0.06 vs 0.22±0.11, p=0.003).

Results showed that immunoreactivity against the S6 band in the soy dosed group was similar to immunoreactivity in the asymptomatic animals in Experiment 3. This suggested both types of rats, asymptomatic BBdp fed SPI and the current BBdp rats receiving soy antigens in infancy, showed a similar "protective" higher immune reactivity against S6. These results also suggested that early oral dosing with soy "diabetogenic" antigens delayed diabetes onset and protected ~30% of the animals from becoming

Table 11. Comparison of differences in immunoreactivity of soymeal fractions following early oral dosing with soymeal.

				Asymptomatic				Diabetic			
						p value				*p* value	
Band	Diet	Oral Tx Day 4-7	n	Freq.	Absorbance	Freq.	Absorbance	Freq.	Absorbance	Freq.	Absorbance
S6	Soymeal	Soymeal in Pregestimil	15	7/10	0.18±0.13	0.02	0.02	3/5	0.14±0.13	0.50	0.92
	Soymeal	Pregestimil	15	1/8	0.03±0.07			3/7	0.13±0.16		
S8	Soymeal	Soymeal in Pregestimil	15	7/10	0.17±0.12	0.62	0.80	2/5	0.08±0.11	0.15	0.03
	Soymeal	Pregestimil	15	6/8	0.15±0.09			6/7	0.23±0.11		
S10	Soymeal	Soymeal in Pregestimil	15	3/10	0.08±0.14	0.38	0.26	1/5	0.03±0.06	0.05	0.004
	Soymeal	Pregestimil	15	1/8	0.03±0.07			6/7	0.23±0.11		
S13	Soymeal	Soymeal in Pregestimil	15	1/10	0.02±0.08	0.56	0.53	1/5	0.05±0.12	0.68	0.83
	Soymeal	Pregestimil	15	0/8	0.00±0.00			1/7	0.04±0.11		

diabetic. Early oral dosing resulted in dark S6 bands (protective) in asymptomatic rats and decreased frequency and intensity of the S10 bands (diabetes-related) in overt diabetic rats.

Experiment 6 Pre-absorbing Antibodies from BB Rat Serum with Rat Insulinoma Cells, RINm5F: A Preliminary Study

In this experiment the cross reactivity of soy antibodies with antigens associated with rat insulinoma (β cell tumour line), RINm5F cells, was analysed. Antibodies in sera from different types of BB rats were absorbed with RINm5F cells, then applied to a nitrocellulose blot of SPI protein extract and the presence of rat IgG and IgA antibodies were measured.

Results indicated that the intensity of almost all bands in the SPI extract was decreased using pre-absorbed serum. This decrease in intensity was more visible using anti-rat IgG-HRP as the secondary antibody compared with anti-rat IgA-HRP antibody. Regardless of which type of serum was used, the most affected bands in the SPI extract were the S2 and S3 bands. In BBc sera which were pre-absorbed, the intensity of the S13 band was significantly decreased (0.18 ± 0.03 vs 0.28 ± 0.03, $p<0.001$). The S6, S8 and S10 bands were not affected by pre-absorption (Fig. 20).

DISCUSSION

There is now evidence that spontaneous diabetes in BBdp rats and NOD mice is to a large extent, food-induced (>80%). The incidence of diabetes is the highest in animals fed mainly plant based diets. Analyses have shown that the major diabetogenic protein components in these diets are wheat gluten (Scott et al. 1988) and soy (Hoorfar et al. 1991) in BB rats. Although sometimes cow milk proteins can act as food diabetogens affecting BBdp rats (Scott et al. 1996a; Scott 1994b; Elliott and Martin 1984), high diabetes incidence in rats fed skim milk based diets is not seen consistently. Wheat gluten and soy have also been reported to be diabetogenic in NOD mice (Hoorfar et al. 1993).

IDDM is now generally classified as an autoimmune disease (Bach, 1994). By definition, autoimmunity is a failure of the immune system to maintain tolerance to its own tissues with subsequent destruction of self structures. The critical elements of this breakdown of self-tolerance include class II MHC-linked susceptibility genes, non-HLA genetic factors, possibly related to T-cell receptor and immunoglobulin genes; and as yet unidentified environmental agents operating by local inflammation or by molecular mimicry (Sinha, Lopez and McDevitt 1990). It has been suggested that diabetes is a Th1-cell mediated disease (Rabinovitch, 1994) and a recent report indicates that a protective diet shifts the predominance of Th1 to Th2 cells in the pancreas (Scott et al., In Press, 1997). Most of the β-cell autoantigens detected thus far have been identified initially by demonstrating antibodies against them in sera of diabetic rodents or patients. An antigen of molecular mass 64 kDa was detected by immunoprecipitation of islet cell lysates (Baekkeskov et al. 1982), and was biochemically characterized as glutamic acid decarboxylase (GAD). However, autoantibodies likely play a small role in the

pathogenesis of IDDM and although B-cells are found among the cells that infiltrate the islets, antibody production seems to be a secondary event following T-cell mediated destruction of β-cells (Bach 1994). Nonetheless, altered antibody response also reflects changes in T helper cells which are required for the production of antibody.

The physiological antibody response to foods has been studied by many investigators. The results show that it is normal to make IgG antibodies to food proteins. There are, however, substantial differences in the oral immunogenicity of various food antigens. Some of the differences in antibody levels are likely to be related to the amount of antigen consumed, and some food antigens seem to have a higher intrinsic immunogenicity than others. Depending on the age of the subjects, site of antigen presentation and which cells are encountered and active at the time antigen confronts the immune system, the outcome of the immune response may differ.

Previous reports indicated a relation, in both BB rats (Hoorfar et al. 1991, Atkinson et al. 1988, Brogren et al. 1989) and NOD mice (Hoorfar et al. 1993), between soy and diabetes. The present work is focused on soy and we believe this is the first report in which soy diabetes related protein fractions have been partially characterized.

Experiment 1 Preliminary Identification of Immunogenic NIH and Soy Proteins

The gel electrophoresis results using soy-based powder infant formulas (Isomil and Prosobee) showed fewer proteins and a predominance of low molecular mass components. Because only low molecular mass proteins such as S13 were observed in powdered formula, we continued the western blotting analysis using liquid formulas. It may be possible to obtain a more detailed band analysis by changing pore size of the gel, but we did not pursue this option in these studies. In the case of the liquid infant formulas, more of the high molecular mass proteins were left on the top of the gel which could explain the

lower amount of proteins in the gel compared with SPI or soybean meal protein fractions.

To compare NIH and soy antibodies in animals at various levels of risk for developing diabetes, we chose BBc, BBdp and BBd animals over the age range during which diabetes develops (62-138 d) in the Health Canada colony. Rats in different groups were randomly selected from the colony and the mean ages were similar (BBc, 92 d; BBdp, 85 d; BBd-U, 82 d; BBd-T, 102 d).

It was apparent that certain bands from Western blots in both NIH and soy extracts were diabetes-related; N6, N7, N8 for NIH and S6, S8 and S10 for soy. A crude comparison indicated that some bands in the NIH extract had similar molecular mass to certain bands in the soy extracts: for example, the N6 band (~50 kDa) in NIH resembled the S6 band (~50 kDa) in soy, the N7 ≈ S8 (~41 kDa), N8 ≈ S10 (~35 kDa) and N11 ≈ S13 band (~19 kDa). However, NIH also contains diabetogenic wheat gluten and milk. Thus, although it is not surprising to see the "soy pattern" in the NIH extract as NIH contains soymeal, it is not possible to equate directly the NIH and soy diabetes-related bands.

Results from this analysis showed that the S6 and S13 bands were less intense and less frequent in diabetic rats compared with control animals and suggested that the presence of these bands was characteristic of animals that do not develop diabetes. The presence of a higher immunoreactivity against the S10 band was characteristic of diabetic rats and S10 antibodies were rarely seen in control rats. Of all the bands identified as diabetes-related, the S10 (and possibly N8) band was the most clearly diabetes-related. A link between the S8 band and diabetes was only apparent as a decrease in intensity when BBd rats were treated with insulin. The two clearest associations with diabetes were higher S10 and low S13 antibody binding in diabetic vs control BB rats. This study

suggested we had identified a diabetes-related and a protective band pattern.

Based on values in the literature (Burks et al. 1989; Wolf, Peterson and Schaer 1992; Burks, Brooks and Sampson 1988) and crude comparison of molecular mass, the identity of the diabetes-related soy proteins can be tentatively assigned as follows: S6 resembles the β subunit of the 7S β-conglycinins, the S8 fraction is similar to the A3 subunit of the acidic subunit of the 11S glycinins, the S10 fraction may be the A1a, A1b or A2 subunits of the acidic subunit of the 11S glycinins and the S13 fraction may be the basic subunit of the 11S glycinins.

When diabetic BB rats were treated with insulin, the Western blot pattern was not the same as that seen in untreated BBd rats (Table 1). The major difference was in the N8 and S10 antibody levels which were decreased in insulin-treated BBd rats compared with untreated BBd rats. This suggested that insulin treatment somehow restored more normal reactivity against the S10 (and N8) proteins indicating a possible reversal or partial recovery from the usual immunodeficient state in BBdp rats (Rossini et al. 1993). We cannot explain this phenomenon, however, this decrease in the immunoreactivity could be attributable to a suppressive effect on antibody production by enhancing certain activated T suppressor cells which have insulin receptors on the surface, or might reflect an effect on oral immune tolerance to the S10 proteins. Another difference was observed with respect to the S8 band which was less intense in BBd rats and reached significance in BBd-T (insulin treated) rats compared with BBc or BBdp rats. The relationship between the S8 band and diabetes was less clear than that of the S10 band and was primarily seen when comparing insulin-treated BBd values to BBc and BBdp.

Experiment 2 Characterization of the Development of Soy Antibodies with Age in a Cross-Sectional Study of BB Rats

The results of Experiment 2 confirmed and expanded the findings from Experiment 1 and showed that the S6, S10 and S13 bands were important bands for differentiating control and diabetes-prone rats. With respect to the S6 band, BBdp rats developed antibodies later than did BBc rats, such that very few BBdp rats at 45 d had the S6 antibodies compared with BBc rats. More of the BBdp rats lacked antibodies to the S6 at an early age. This decreased reactivity against the S6 band in BBdp rats compared with BBc confirmed the results in Experiment 1. It seems that BBc (non-diabetes-prone) animals, which do not develop diabetes commonly show more reactivity against the S6 band. The S10 antibodies were seen in most BBdp rats during the pre-diabetic period at 45 d and 70 d of age, but were much less prevalent in BBc rats. This suggested that S10 antibodies might reflect diabetes development and is in keeping with the results of Experiment 1 showing a progressive increase in the intensity of the S10 staining as one goes from BBc→BBdp→BBd-U rats. S10 reactivity was the most clearly diabetes-related band. Previous reports also suggest that BBdp rats have increased levels of antibodies to milk and wheat (Scott, Cloutier, Souligny, Riley, Hoorfar and Brogren 1989) and similar results have been reported in NOD mice for the milk protein, BSA (Beppu et al. 1987).

The S13 antibodies appear later in life, after 45 d in BBc rats but are virtually absent in BBdp rats and are therefore characteristic of control (non-diabetes-prone) rats. The S8 band showed no differences in frequency between BBc or BBdp rats at different ages. Therefore, because the S13 was BBc related and the S8 showed no difference between BBc and BBdp rats in contrast to the differences in BBc vs BBdp for the S6 and S10 bands, these data suggested that the S6 and S10 bands were more important predictors

of diabetes. IgG antibodies were more predominant and less IgA antibody reactivity was seen in this experiment which led us to focus solely on IgG antibodies in the next experiments.

Therefore, in keeping with the finding from Experiment 1, that the S10 was diabetes-related, the results of this time course study showed BBdp rats were more likely to have antibodies to S10 at 45 d and 70 d. This suggested reactivity against S10 might reflect early destruction of islet β-cells and may be a diabetes-related marker of pathogenesis. Overt islet cell destruction likely begins by ~50 d when small numbers of infiltrating mononuclear cells are first visible by light microscopy (Hanenberg, Kolb-Bachofen, Kantwerk-Funke and Kolb 1989); by 70 d, BB rats are beginning to develop diabetes and 10-15 % of animals will have developed overt diseases. These results confirmed the findings from Experiment 1 and indicated that there were differences in soy antibody levels present during the crucial pre-diabetic period when insulitis is first visible. This experiment also revealed a trend such that S6 antibody levels were lower in BBdp than in BBc rats as was seen in Experiment 1.

Experiment 3 A Prospective Study of IgG Antibodies in BBdp Rat Sera that Bind SPI Proteins: Effect of Various Soy Diets

Previous results in our laboratory from five experiments indicated that diabetes-prone BB rats fed a defined diet in which the sole source of protein was soybean meal showed a mean diabetes frequency of 45±8% (SD), considerably higher than the usual negative control diet with casein or hydrolysed casein (HC) as the sole protein source (12 ±5%, n=8 experiments). Others have also observed that soy-based diets are moderately diabetogenic, resulting in 38% (Atkinson, Winter and Skordis et al. 1988) and 60% (Brogren, Hoorfar and Buschard 1989) diabetes incidence in the BB rat.

Soybean meal is a much less refined preparation compared with the soy protein isolates (SPI) used to make soy-based infant formulas. Although our preliminary studies show that diets containing SPI-based infant formulas are less diabetogenic than soymeal diets fed to BBdp rats, it was important to define more clearly the diabetogenic activity of SPI. The present experiment showed that substantial diabetogenic activity remained in SPI despite processing. This is important because ~20-25 % of infants in North America are fed soy-based infant formulas. Furthermore, there are indications that infants fed soy formula are more at risk of developing autoimmune diabetes or autoimmune thyroiditis (Fort, Lanes and Dahlem et al. 1986; Fort, Moses and Fasano et al. 1990). The American Academy of Pediatrics revised its guidelines recently indicating that infants at risk of developing diabetes should not be fed soy formula in the first year of life (Drash et al., 1994). In this experiment, the diabetogenicity of various soy products including liquid and powdered soy-based infant formulas, was characterized and found to be dependent on the individual soy protein source. The diabetes incidence in BBdp rats fed a powdered soy-based infant formula diet (35%) was higher compared with animals fed liquid soy-based infant formula diet (19%). The soybean protein isolate used to make powdered soy formula is a mildly hydrolyzed form of the isolate used in the liquid soy formula. This was evident from SDS-PAGE gels of the various soy protein sources which showed fewer protein bands in powdered compared with liquid soy-based infant formulas (Figure 1). This result suggested that the mild hydrolysis process used to prepare the powdered formula did not necessarily destroy all of the diabetogenicity of SPI and inferred that the diabetogenic potential may reside in small peptides.

The different diabetogenicity of soy formulas and other products (SPI, soymeal) could be the result of differences in processing and how the immune system reacts to the

resulting products. In the manufacture of infant formulas, there are large variations in heat-treatment of the products, resulting in differences in solubility and digestibility of the proteins in the formulas (Rudloff and Lonnerdal 1992). Heat-treatment may, in fact, be important in determining the immune response following feeding of milk and soy products. It is also likely that there is batch to batch variation in levels of food diabetogens in addition to processing effects. A critical role of heat denaturation for the immunogenicity of whey proteins was demonstrated by Enomoto, Konishi, Hachimura and Kaminogawa (1993) who showed that feeding whey protein to mice induced oral tolerance. This was shown by feeding heat-treated whey protein which induced oral tolerance. It may be possible that the feeding of soy-based liquid infant formulas with more intact proteins produce a primary tolerance in these animals, a point we investigated using soymeal in Experiment 5.

Different IgE specific and IgG specific antibody responses were reported to the soy protein isolates used in liquid and powdered soy-based formulas (Burks, Butler, Brooks et al. 1988). ELISA results showed that specific IgE and IgG antibodies to liquid infant formulas were significantly higher than to powdered formulas. SDS polyacrylamide gel electrophoresis showed that the liquid formula had bands representing 7S (β-conglycinin) subunits and the acidic and basic subunits of the 11S proteins. Only small molecular weight compounds were seen in the powdered soy infant formulas. Thus, there may be a difference in the allergenicity and by inference, the antigenicity of liquid and powdered infant formulas (Barbashov 1991). The interrelationship of the timing of introduction of formulas, cow milk, and solid foods into the infant's diet makes it difficult to disentangle the independent effects of these dietary exposures on development of IDDM in humans (Kostraba 1994). Thus, the present studies provide new insights into potential food (soy)-

autoimmune disease links.

Previous studies indicated that hydrolyzing soy protein did not consistently result in lower diabetes incidence suggesting that the soy diabetogenic activity can be retained in smaller peptides (Scott 1995b). There are other indications that soy-based diets are associated with slightly delayed onset and result in mild to moderate diabetes incidence in high-incidence NOD mouse colonies (Hoorfar et al., 1993; Scott 1995b). An SPI diet produced ~35% diabetes incidence by 30 weeks of age compared with ~50% incidence in NOD mice fed a cereal-based, (positive control) Purina chow 5058 diet (Purina Mills, Richmond, IN). Hoorfar and colleagues found a soybean meal diet produced 45% diabetes incidence in high-incidence NOD mice (Hoorfar, Buschard and Dagnaes-Hansen, 1993). As in the BB rat, an HC-based, Pregestimil® (Mead Johnson, Evansville, IN) diet may be considered diabetes-retardant, producing no diabetes at 30 weeks of age (Coleman, Kuzawa and Leiter, 1990). Overall, these data suggest that soybeans contain a diabetogenic component which may be decreased but not necessarily destroyed during food processing. The current results confirm and expand this contention, showing that processing may alter diabetogenicity markedly.

Western blot analyses of prospective serum samples from BBdp rats fed different soy protein sources showed that the SPI fed group, which had the highest diabetes incidence (41%), also showed the most consistent patterns of antibody binding to the bands identified in experiments 1 and 2. The lack of consistent results seen when using sera from rats fed powder or liquid soy formula, appeared to be linked to the use of the SPI extract for all Western blot analyses regardless of the diet eaten. That is, when the extract was matched to the soy protein source eaten by the animals, results were more consistent. Serum from SPI-fed rats submitted to blotting of SPI extracts showed band

patterns that were more consistent and interpretable. Therefore, our major interpretation in this experiment is based on serum from SPI-fed rats blotted against SPI proteins. These data suggest that it may be important to evaluate food derived immune reactivity using the exact dietary protein source (Mowat 1987).

The differences in the immunoreactivity of the bands at 45 d, a crucial time in the pre-diabetic period, showed a predictive pattern comparing asymptomatic animals with pre-diabetic rats. The pattern suggested low S6 reactivity and high S10 reactivity (Figure 8) can be used as markers of animals destined to become diabetic in later life. Although reactivity against the S8 band correlated with diabetes incidence at 45 d in pre-diabetic animals (Figure 8), antibodies against this band were not useful in differentiating which diabetes-prone rats would become diabetic as shown in experiments 1 and 2. In the liquid formula-fed animals there were only three animals in the pre-diabetic or diabetic group which made comparisons impractical.

Comparing immunoreactivity against the S6 band at 45 d among different diet groups with different diabetes frequency showed that there is a positive correlation between the incidence of diabetes and frequency of the reactivity against this band. At 45 d, in asymptomatic rats, as the incidence of diabetes increased more animals reacted to the S6 band ($r=0.95$, $p=0.05$) which again was similar to the results observed in experiments 1 and 2 (Table 1, Figure 5). These results suggested reactivity against this band is related to resistance, identifying animals that are at low risk of becoming diabetic. This finding is contrary to our expectations that enhanced antibody levels to selected soy proteins would indicate potentially diabetogenic proteins. It suggests that animals with less immunodeficiency and a more normal functioning gut immune system may avoid diabetes (Stephen, Thompson and Staines 1990).

S10 band frequency was positively correlated with incidence of diabetes in pre-diabetic rats at 70 d (r=0.92, p=0.07) suggesting again this band was diabetes-related. The S13 band frequency was positively correlated in asymptomatic animals at 45 d (r=0.98, p=0.02). This indicated that S13 antibodies were more characteristic of asymptomatic rats compared with pre-diabetic rats. However, reactivity against this band was still much lower in BBdp rats compared with BBc animals (Table 1, Figure 5). It is important to note that all diet groups were tested using SPI extract as the source of antigens to which antibodies were bound on the nitrocellulose paper. These data confirmed and expanded the results from experiments 1 and 2, clearly indicating that certain patterns could be identified as diabetes-related. For example, animals with high reactivity at 45 d to S10, low S6 and S13 band frequency were more at risk of developing diabetes (Figure 8).

The finding of these time-dependent patterns is in keeping with the suggestion that timing and duration of exposure to diabetogens is important (Scott et al., In Press 1997). Studies in the BB rat show that even as late as puberty, BB rats can be rescued from development of diabetes by removal of food diabetogens and first exposure at this time to food diabetogens can still induce the disease (Scott and Marliss 1991; Scott et al., In Press 1997).

Experiment 4 Identification of the Immunogenic Soy Antigens Using Sera from Control and Diabetic Humans

Other groups have used similar immunochemical approaches to examine food or target cell antibody levels (Beppu et al. 1987, Scott et al. 1989b). The first observation linking the immune response to a dietary protein to insulin-dependent diabetes was provided by Beppu et al. (1987), who showed that NOD mice had higher levels and higher

frequency of positive serum IgG, IgA and IgM antibodies to BSA than did control mice, as measured by ELISA. Furthermore, diabetic NOD mice had a higher frequency of IgG antibodies to BSA than did non-diabetic NOD mice. In human, Savilahti et al. (1988) found increased levels of IgA antibodies to whole cow's milk and BLG (β-lactoglobulin), and IgG antibodies to BLG, in children with newly diagnosed IDDM. This finding was confirmed in a study of Swedish children with recent onset diabetes compared with age- and sex-matched controls (Dahlquist, Savilahti and Landin-Olsson 1992).

To my knowledge, there are no reported studies of soy antibodies in human diabetic patients. There are however, several reports of IgE antibody responses in patients with soy allergy (Burks et al. 1991; Burks et al. 1992; Burks et al. 1994; Sandiford, Tee and Newman-Taylor 1995; Tsuji, Bando, Kimoto, Okada and Ogawa 1993). In a study by Hvatum, Scott and Brandtzaeg (1992), levels of serum IgA, IgG and IgG subclass antibodies to a variety of dietary antigens were determined by enzyme linked immunosorbent assay in adults with coeliac disease and control adults. Results indicated raised total IgG, IgA, IgG1 and IgG3 antibodies to gliadin but reduced IgG4 antibodies compared with the controls.

In our Ottawa study, results for the SPI extract immunoblots showed low S6 and S13 antibody frequency and absorbance in diabetic patients compared with controls. Of particular note, S10 band frequency was higher in diabetic patients compared with control individuals. There was a trend showing lower S13 immunoreactivity in diabetic patients again suggesting that reactivity against S13 is protective. Therefore, it is remarkable that in spite of the small numbers and incompletely matched controls, this experiment showed that S10 band antibodies were diabetes-related as was shown in the first three animal experiments. This preliminary study suggested therefore that human

patients had similar patterns to those seen in diabetic BB rats. This result was further confirmed in the more detailed analysis of the sera from Finland.

In the study of Finnish blood samples, both the IgG and IgA responses against the S10 band were significantly higher compared with age and sex-matched controls. S6 and S13 bands were less frequent and less intense in diabetic patients. Overall, the immunoreactivity against the S10 band was increased in diabetic groups in both the Ottawa and Finland studies. This finding, in two separate studies of human diabetic patients, was in keeping with the results from studies in BB rats and strongly suggested that reactivity to these soy proteins is in some way diabetes-related.

Experiment 5 Effect of Early Oral Dosing with Soybean Meal on Diabetes Development in BB Rats

Autoimmune disease is by definition a breaking of tolerance to self antigens. Suppression of immune responses following orally fed protein antigens (oral tolerance) has long been recognized in laboratory animals (Wells, 1911). Studies in mice have shown that feeding of the antigen during the early neonatal period can lead to priming of the immune system, while feeding at a later age induces tolerance (Strobel and Ferguson 1984). Declining IgG antibody response to dietary protein with age despite continuing exposure suggests age-dependent development of tolerance also in human (Korenblat et al. 1968, Kletter et al. 1971a). This may be related to maturation of the immune system as antibody response is depressed if the first exposure to dietary protein is postponed (Kletter et al. 1971b). Mechanisms associated with the induction and maintenance of oral tolerance have been studied in laboratory animals and recently in human (Weiner, Friedman, Miller, Khoury, Santos, Sayegh, Nussenblatt, Trentham and Hafler, 1994). There is evidence for the role of generation of active suppression in oral tolerance (Mowat

1987) and some have suggested this might be mediated by induction of antigen specific suppressor T-cells (Richman, Chiller, Brown, Hanson and Vaz, 1978). Induction of tolerance to diabetes-related antigens either by injecting β-cells directly into the thymus or inducing oral tolerance to insulin has been shown to inhibit diabetes and clinical trials using oral insulin are currently underway. If as we suspect, the food diabetogens are peptides similar in nature to β-cell related peptides and they induce some form of cross reactivity reactivity or enhanced β-cell antigenicity, it might be possible to induce tolerance by orally dosing BBdp rats with these diabetogenic foods very early in life. The time period between day 4 and 7 is when oral tolerance first appears in rats (Mowat 1987). In order to test this proposition, BBdp rats were dosed twice daily with mg amounts of diabetogenic soybean meal between 4 to 7 d of age, and the animals were weaned onto the same diet (i.e., soymeal-based) to which they were exposed in infancy.

This early exposure delayed the onset of diabetes by 29 days ($p<0.05$) and reduced diabetes frequency from 47 % to 35 %. This pattern of delayed onset and partial protection (Figure 19) has been seen in 6 separate experimental groups of BB rats dosed orally early in life with other food diabetogens in our laboratory (Scott and Rowsell 1995; Rowsell MSc. thesis, 1996). On average, ~37 % of diabetes cases are prevented by this regime. These results suggest that diabetes can be delayed or even avoided in some individuals by very early oral exposure to food diabetogens, likely reflecting an important role for the gut immune system (Scott et al., 1996; Stephen et al., 1990).

Western blot analysis indicated that orally dosed (potentially protected) animals had higher reactivity against the S6 band compared with animals which were not orally dosed with soybean meal ($p=0.03$). This again supported the previous results that enhanced reactivity against the S6 band is protective and showed that these animals have a similar

pattern to that seen in asymptomatic BBdp rats. When animals were divided in two groups of asymptomatic and diabetic rats at the end of the study, analysis showed lower S10 reactivity in orally dosed animals that became diabetic while higher S6 reactivity was observed in asymptomatic rats that were dosed orally. Therefore, some rats became diabetic without having antibodies to S10. This contradiction reflects the fact that the oral dosing regime and/or concentrations are not optimized and this treatment is only partially effective at this time. However, S10 reactivity was again seen in most diabetic rats that were not orally dosed as we observed in experiments 1-3. As the only two bands showing significant differences between orally dosed and non-dosed groups were S6 and S10, S6 in asymptomatic and S10 in diabetic rats, this again supported our conclusion that S10 is diabetes-related and S6 reactivity is protective.

Experiment 6 Pre-absorbing Antibodies from BB Rat Serum with Rat Insulinoma Cells, RINm5F: A Preliminary Study

In this preliminary study, anti-soy antibodies in BB rat sera were pre-absorbed with RIN cells and the sera were applied to blots of SPI extracts to compare the intensity of binding. The results indicated a decrease in the intensity of binding in almost all immunoreactive bands when using pre-absorbed sera compared to non-absorbed sera. This result has at least two interpretations: (i) the RIN cell pre-absorption non-specifically absorbed out a portion of all antibodies or (ii) there was cross reactivity among several soy proteins and RIN cell proteins. These possibilities could be distinguished by observing changes in non-diabetes-related (e.g. foods other than wheat, soy, milk) food antibodies to determine if there was a general non-specific absorption of all serum antibodies. The fact that binding to a few soy protein bands was unchanged suggests that not all antibodies were absorbed.

In summary, it has been found that soy is diabetogenic in animal models of type I diabetes (Hoorfar et al. 1991, Atkinson et al. 1988, Brogren et al. 1989 and Hoorfar et al. 1993). The results of Experiment 3 support this finding and further suggest that some types of food processing, particularly of soy-based infant formulas, can decrease but not necessarily abolish diabetogenicity. However, to date, it has not been shown which component(s) are diabetes related. The analyses reported in this thesis show that the most interesting protein fraction of soy was the S10 fraction which was a highly diabetes-related band and reactivity against this band was associated with increased risk of developing diabetes. This fraction has a molecular mass of ~35 kDa and (Burks et al. 1989, Wolf et al. 1992, Burks et al. 1988) is likely the A1a, A1b or A2 subunits of the acidic subunit of 11S glycinin. However, these data only suggest that the diabetogenic components of the S10 soy protein fractions were indeed A1a, A1b, or the A2 subunits of the acidic subunit of 11S glycinins. Actual identification of these proteins could be achieved by adding purifired soy protein standards (if available) and comparing Rf and molecular mass under the same separation conditions. Another means of identification would require using monoclonal antibodies to individual glycinin subunits as secondary antibodies on Western blots.

It has also been shown previously that the major insulin modulating components of soybean protein are the acidic A1a and A2 polypeptides of glycinin (Minami et al. 1990). It has been reported that insulin autoantibodies could be involved in autoimmune disease as initiators of the disease process or as effectors causing the actual immune destruction. When autoimmune disease occurs, they may also be present as markers (Greenbaum and Palmer 1996).

Results in the cross sectional study again showed high S10 reactivity in BBdp

animals compared with BBc in the crucial prediabetic period around 45 and 70 d. However, because this was a cross sectional study in which we did not determine final diabetes incidence, it was not possible to identify which of the BBdp rats in our analysis would have developed diabetes at a later age. There was high S6 reactivity in BBc animals (which never develop diabetes) compared with BBdp rats.

To further analyse these results, a study was carried out in which prospective blood samples were collected at 45 d, 70 d and 149 d or at the time of diagnosis of diabetes from each animal; at the end of the study animals were grouped as asymptomatic or diabetic. Remarkably, analyses of the results from these animals also showed high S6 and S13 reactivity in asymptomatic animals and high S10 reactivity in diabetic rats. It is noted that the S13 reactivity was relatively low even in asymptomatic rats as these animals are BBdp rats and reactivity against the S13 band is characteristic of BBc (non-diabetes-prone) rats. Therefore, these results again indicate that S6 and S13 reactivity is associated with protection and S10 reactivity is highly associated with diabetes.

We can use reactivity of these bands in BBdp animals in the pre-diabetic period, at 45 d, as markers to predict which animals are destined to become diabetic. The overall pattern of no S13 reactivity, low S6 and high S10 reactivities was found in ~90 % of BBdp rats that became diabetic. This suggested that there is an as yet unidentified soy-immune system interaction that is important in diabetes pathogenesis in BBdp rats. Unexpectedly, the results also indicated that development of antibodies against certain soy proteins was characteristic of animals that never develop diabetes (BBc rats; S6, S13 reactivity). Furthermore, among BBdp rats that remain diabetes-free (asymptomatic), this protective reactivity against certain soy proteins was also seen although to a lesser extent.

To find out if these results were applicable in diabetic patients, studies were carried

out in two sets of blood samples from newly diagnosed diabetic children from Ottawa and Finland. In both studies, high S10 reactivity and low S6 and S13 reactivity were seen in diabetic patients compared with control human subjects showing remarkable similarity with the patterns observed in control and diabetic BB rats.

To examine potential mechanisms involved, in soy-induced diabetes, two experiments were carried out: (i) early oral tolerizing and (ii) pre-absorbing soy antibodies with RIN cells. The oral tolerizing experiment showed that early oral dosing with soybean meal between day 4-7, before gut closure, and long before the pups were able to eat the mother's solid food, protected some animals against development of diabetes and delayed the onset of the disease. At the time of gut closure in the rat, at about 18-21 d, the immune system is still immature. Therefore, introducing the dietary antigen before gut closure may help with the maturation of the immune system while the immature immune system is more exposed to intact dietary antigen and may produce tolerance. One explanation of this protective effect may be that dietary antigens mimic islet autoantigens.

This possibility was investigated in a preliminary experiment in which soy antibodies were pre-absorbed with RIN cells (surrogate β-cell antigens). The overall reduction in reactivity to some extent showed cross reactivity between food antigens and antigens associated with (RIN) β-cells. However, decreased antibody binding occurred in several soy protein bands suggesting some non-specific binding. Therefore, this approach must be refined to determine if there is molecular mimicry between β-cell antigens and soy diabetes-related proteins (Nickerson, Luthra and David 1991). This experiment could be further refined by using freshly isolated BBdp rat β-cells instead of the transformed RIN cell line. More controls should be included to differentiate non-specific from specific

absorption.

In conclusion, our results suggest overall that (i) soy bean protein sources contain a diabetogenic component, (ii) that component could be among the S10 proteins, A1a, A1b or the A2 subunits of the acidic subunit of 11S glycinin, which have been reported to be insulin modulating, (iii) patterns of soy antibody presence or absence have been determined that show greater (low S6, high S10, low or no S13 reactivity) or much reduced (high S6, S13, low S10 reactivity) risk of developing diabetes in BBdp rats, (iv) similar patterns are also observed in control subjects and newly diagnosed (untreated) children with juvenile diabetes in Ottawa and Turku, Finland, (v) the fact that oral exposure in early infancy can delay and partially protect against diabetes in BBdp rats suggests an important role of a soy-gut immune system interaction.

Therefore, these studies have identified, for the first time, potential diabetes-related soy proteins in BB rats and human patients. However, the exact quantification of the soy proteins was difficult despite using several controls in this semi-quantitative analyses. This work also confirms and expands previous studies from our laboratory (Scott, 1996) indicating that soybean products can contain diabetogenic agents and that these are present in varying degrees in soy-based infant formulas. It is now clear that soy products processed using different conditions can have altered diabetogenic potential. The present results also confirm the previous studies in our laboratory showing that early oral dosing with certain diabetogenic antigens can delay the onset of disease and protect some animals from becoming diabetic. This study supports the contention that diet is an important factor in diabetes pathogenesis and suggests that it is possible to identify, characterize and possibly inactivate food diabetogens in our diet.

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FIGURES

Figure 1. SDS-PAGE gel electrophoregram of NIH and soymeal extracts, tentative band identification. 25-27 μg/lane of proteins extracted from NIH and soymeal were separated using SDS-PAGE and bands were identified using silver staining. Protein bands were numberred sequentially as N1 to N12 for the NIH extract and S1 to S15 for soy extracts from high to low molecular mass. Based on reports in the literature, tentative identity of each band was assigned.

SOY PROTEINS

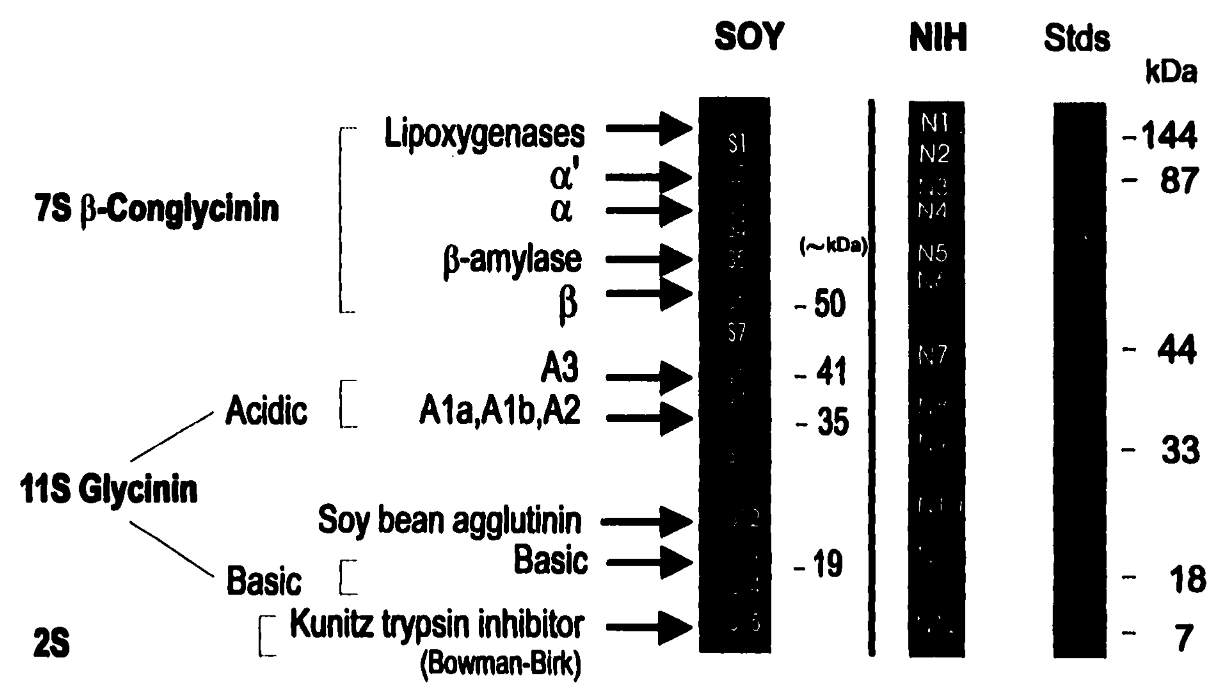
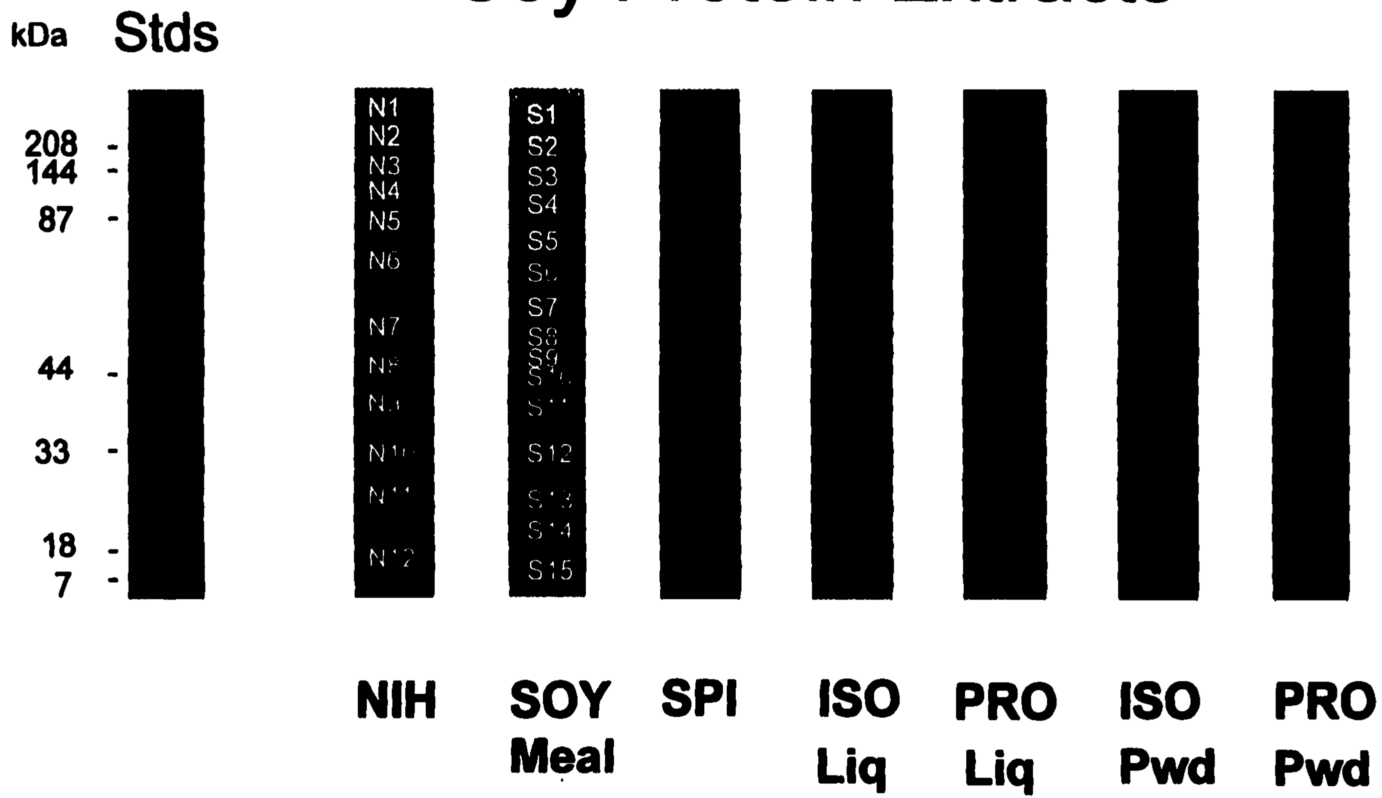


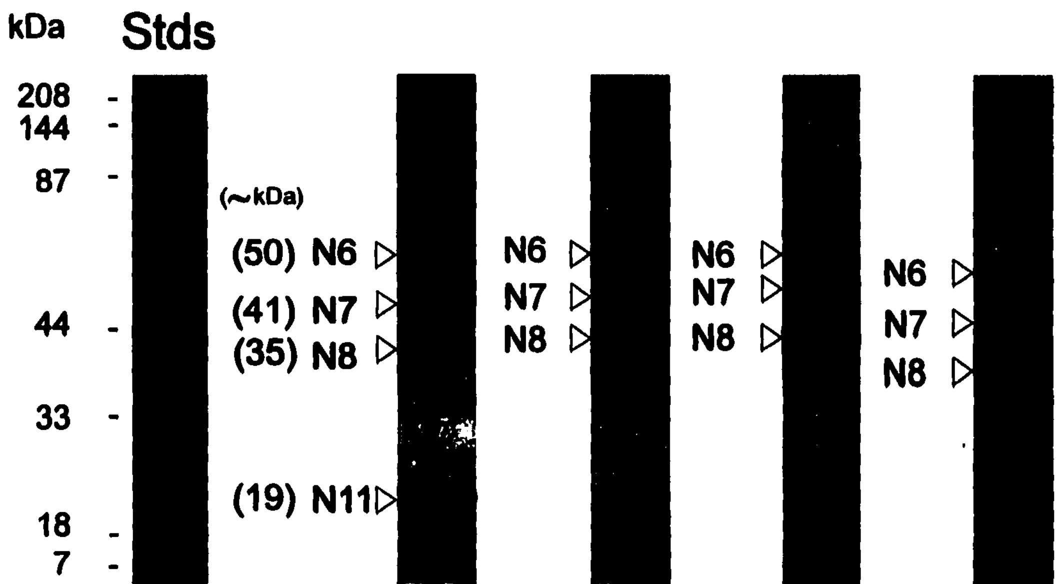
Figure 2. Protein extracts of various soy products separated using SDS-PAGE gel electrophoresis. 25-27 μg/lane of protein extracted from NIH, soybean meal, soy protein isolate (SPI), soy-based liquid (Liq) and powder (Pwd) infant formulas (Isomil, ISO, and Prosobee, PRO) were separated on SDS-PAGE gel electrophoresis and stained with silver reagent as described under materials and methods.



Soy Protein Extracts
kDa
Stds
208
144
87
44
33
18
7
N1
N2
N3
N4
N5
N6
N7
N12
S1
S2
S3
S4
S5
S7
S12
S15
NIH
SOY Meal
SPI
ISO Liq
PRO Liq
ISO Pwd
PRO Pwd

Figure 3. Western blots of diabetogenic NIH protein extracts using BB rat serum. 27 μg per lane of NIH protein extracts were loaded on SDS-PAGE gel electrophoresis, protein fractions were separated, followed by Western blotting using non-diabetes prone (BBc), diabetes-prone (BBdp), diabetic untreated (BBd-U) and diabetic insulin treated (BBd-T) rat sera at 1:1 dilution as the primary antibody and rabbit anti-rat IgG-HRP at 1:800 dilution as the secondary antibody. Protein bands were visualized using diaminobenzidine (DAB) stain solution. The discrepancy of molecular mass of different protein bands in experimental groups is because the lanes were taken from different blots in order to show the overall pattern in each group.

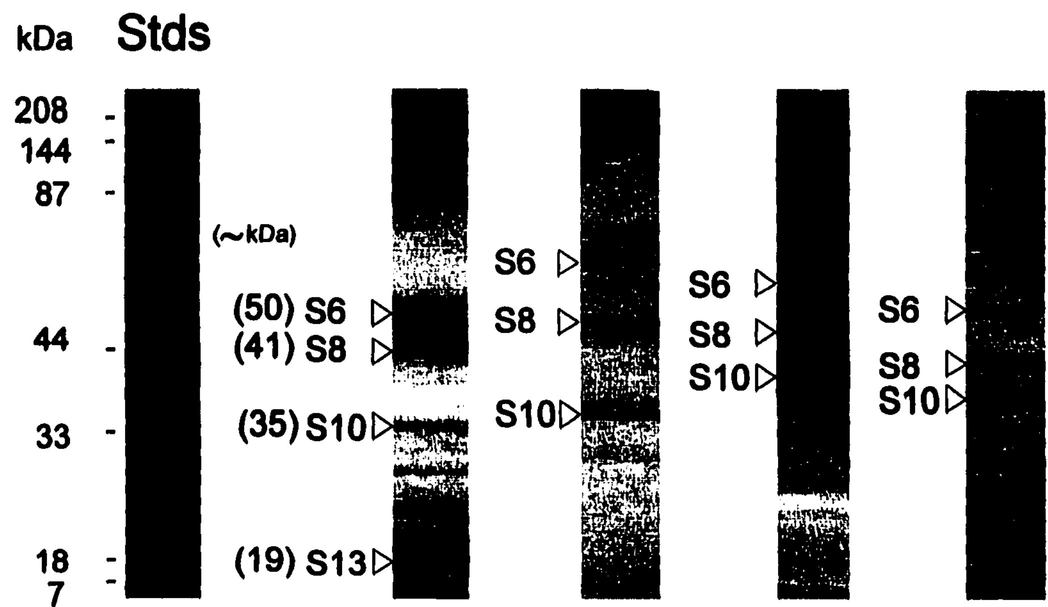
Diabetogenic NIH Diet



BBc BBdp BBd-U BBd-T

Figure 4. Western blots of diabetogenic soy protein isolate protein extracts using BB rat serum. 25.8 µg per lane of soy protein isolate protein extracts (SPI) were loaded on SDS-PAGE gel electrophoresis, protein fractions were separated, followed by Western blotting using non-diabetes prone (BBc), diabetes-prone (BBdp), diabetic untreated (BBd-U) and diabetic insulin treated (BBd-T) rat sera at 1:1 dilution as the primary antibody and rabbit anti-rat IgG-HRP at 1:800 dilution as the secondary antibody. Protein bands were visualized using diaminobenzidine (DAB) stain solution. The discrepancy of molecular mass of different protein bands in experimental groups is because the lanes were taken from different blots in order to show the overall pattern in each group.

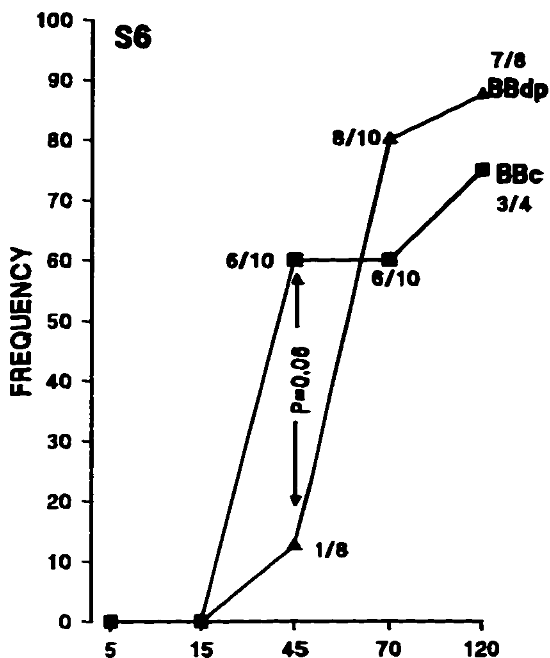
Diabetogenic SPI Diet

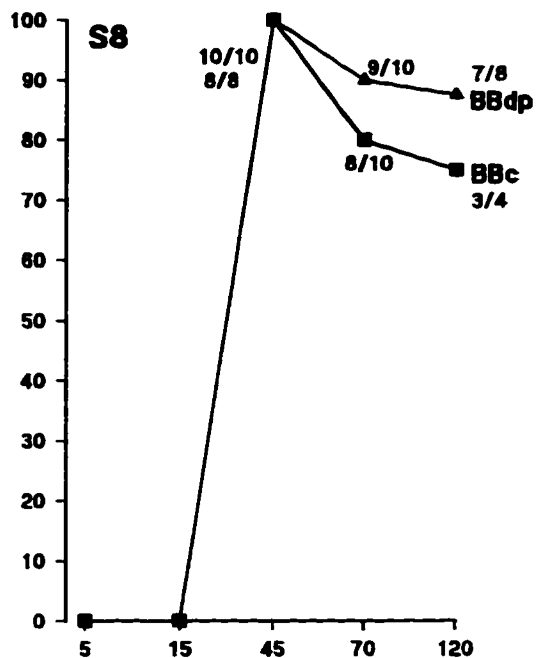


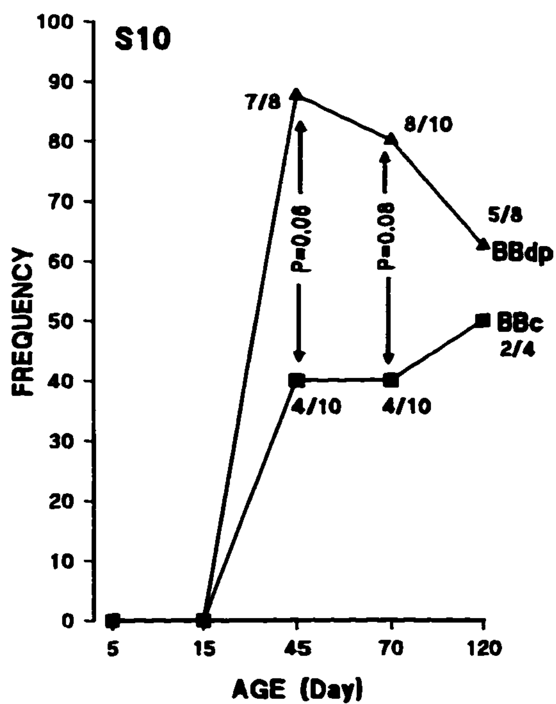
BBc BBdp BBd-U BBd-T

Figure 5. Frequency of IgG antibodies against soy protein isolate (SPI) extracts in BBc (non-diabetes-prone) and BBdp (diabetes-prone) animals in a cross sectional study at 5 d, 15 d, 45 d, 70 d and 120 d. Frequency of four reactive bands (S6, S8, S10 and S13) in both groups of rats were considered. Rats were taken randomly at certain ages from the Health Canada colony.

SPI, IgG







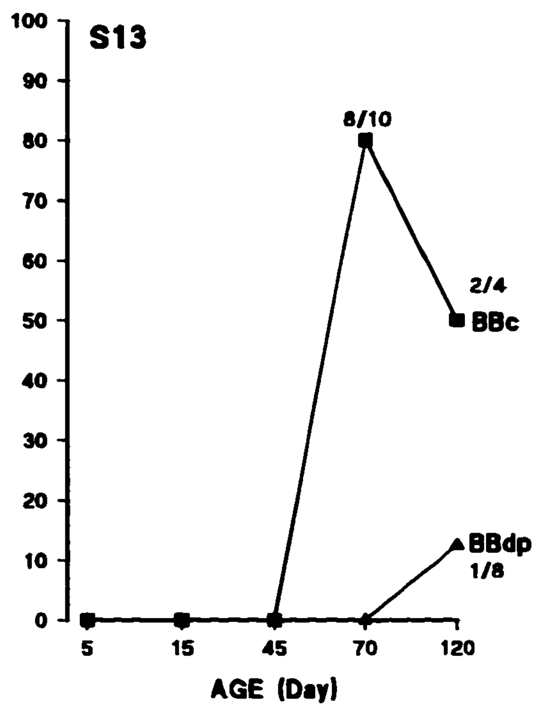
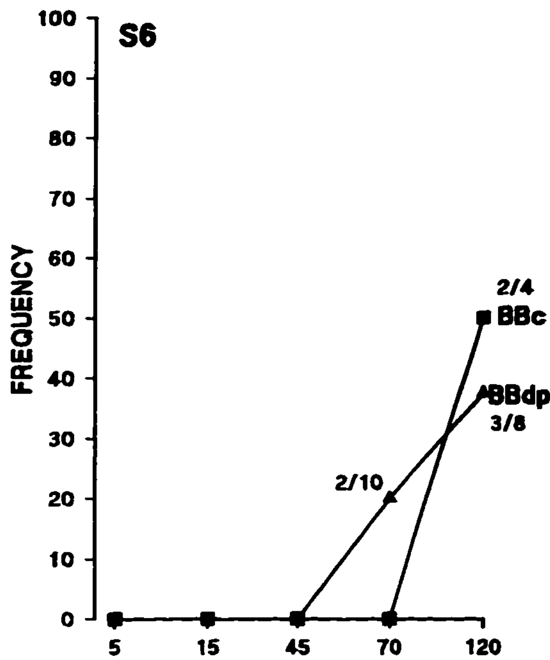
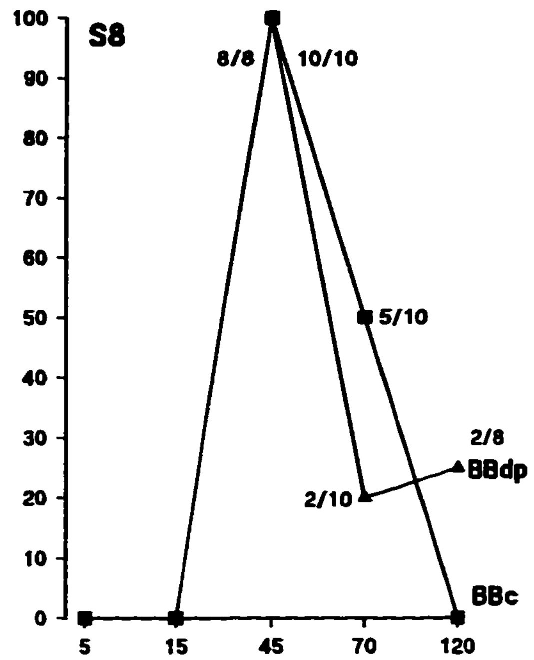
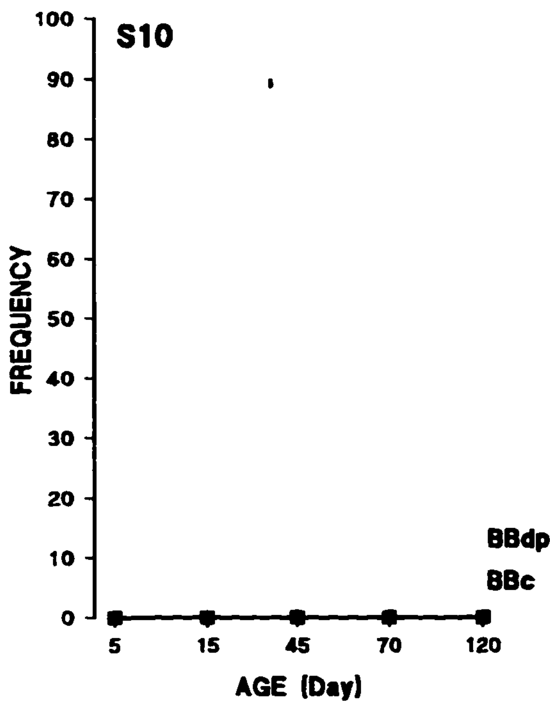


Figure 6. Frequency of IgA antibodies against soy protein isolate (SPI) extracts in BBc (non-diabetes-prone) and BBdp (diabetes-prone) animals in a cross sectional study at 5 d, 15 d, 45 d, 70 d and 120 d. Frequency of four reactive bands (S6, S8, S10 and S13) in both groups of rats were considered. Rats were taken randomly at certain ages from the Health Canada colony.

SPI, IgA







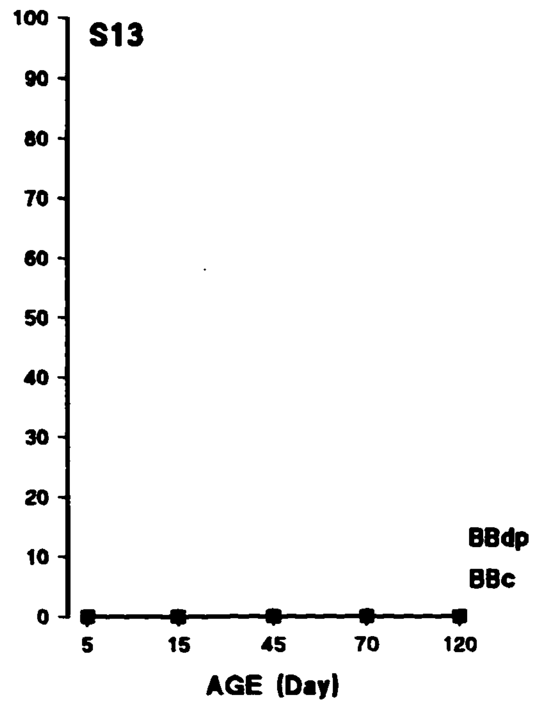


Figure 7. Survival curves showing diabetes incidence in BBdp rats fed different soy diets. Four different soy protein sources were fed to BBdp rats and the development of diabetes was measured by the end of the experiment at 150 day.

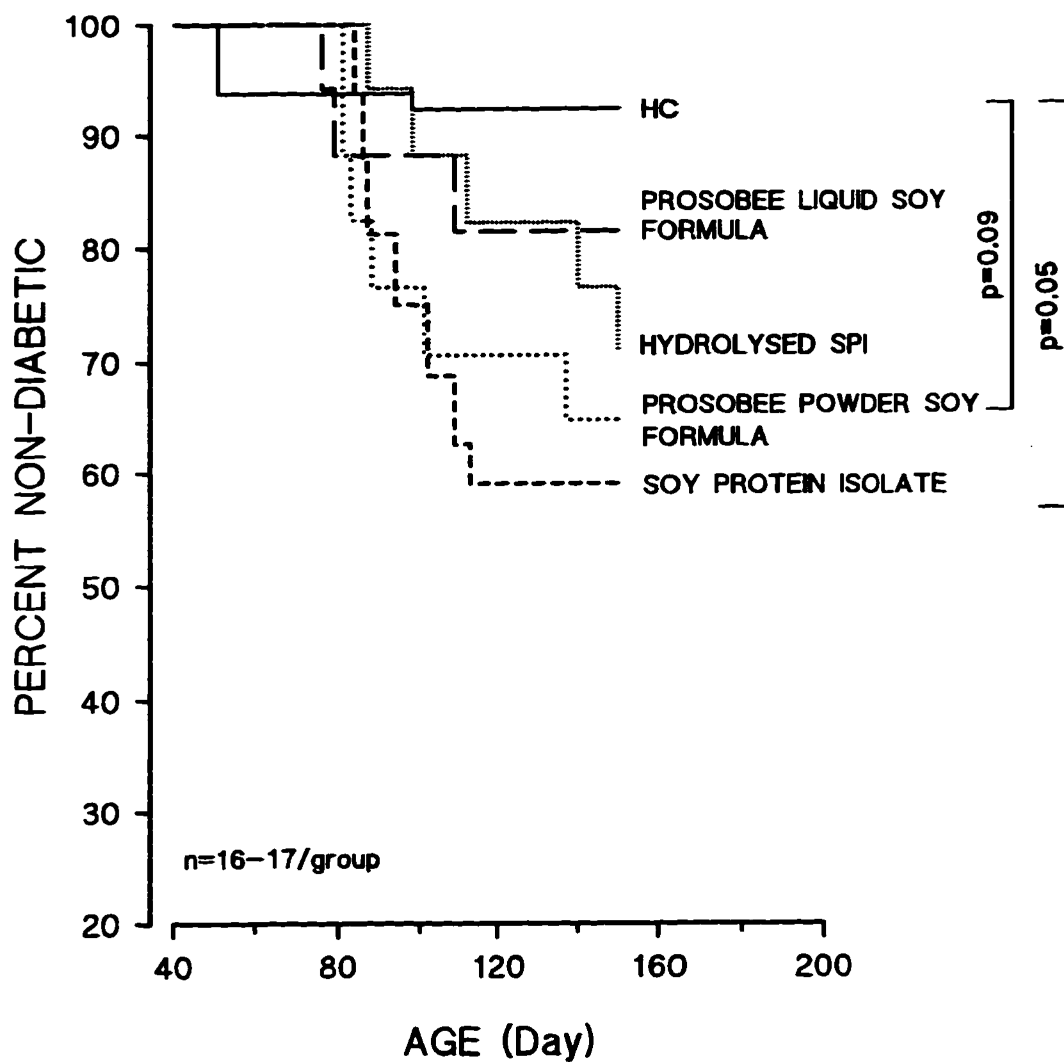

SPI & SOY FORMULA DIETS
PERCENT NON-DIABETIC
100
90
80
70
60
50
40
30
20
40
80
120
160
200
AGE (Day)
HC
PROSOBEE LIQUID SOY FORMULA
HYDROLYSED SPI
PROSOBEE POWDER SOY FORMULA
SOY PROTEIN ISOLATE
p=0.09
p=0.05
n=16-17/group

Figure 8. Frequency of IgG antibody binding to soy fractions in the SPI extract in asymptomatic or diabetes-prone and diabetic BB rats fed SPI diet at various ages. Soy protein isolate proteins were fed to 17 BBdp rats and the outcome of diabetes was measured at the end of the study at 149 d. Based on diabetes outcome, animals were divided into two groups of asymptomatic (n=10) and pre-diabetic or diabetic (n=7). IgG antibodies were measured against the blood samples from these two groups of animals. SPI extract was the antigen and immunoreactive bands were visualized by DAB staining. 96 d is the mean age at diagnosis of diabetes in animals that developed diabetes.

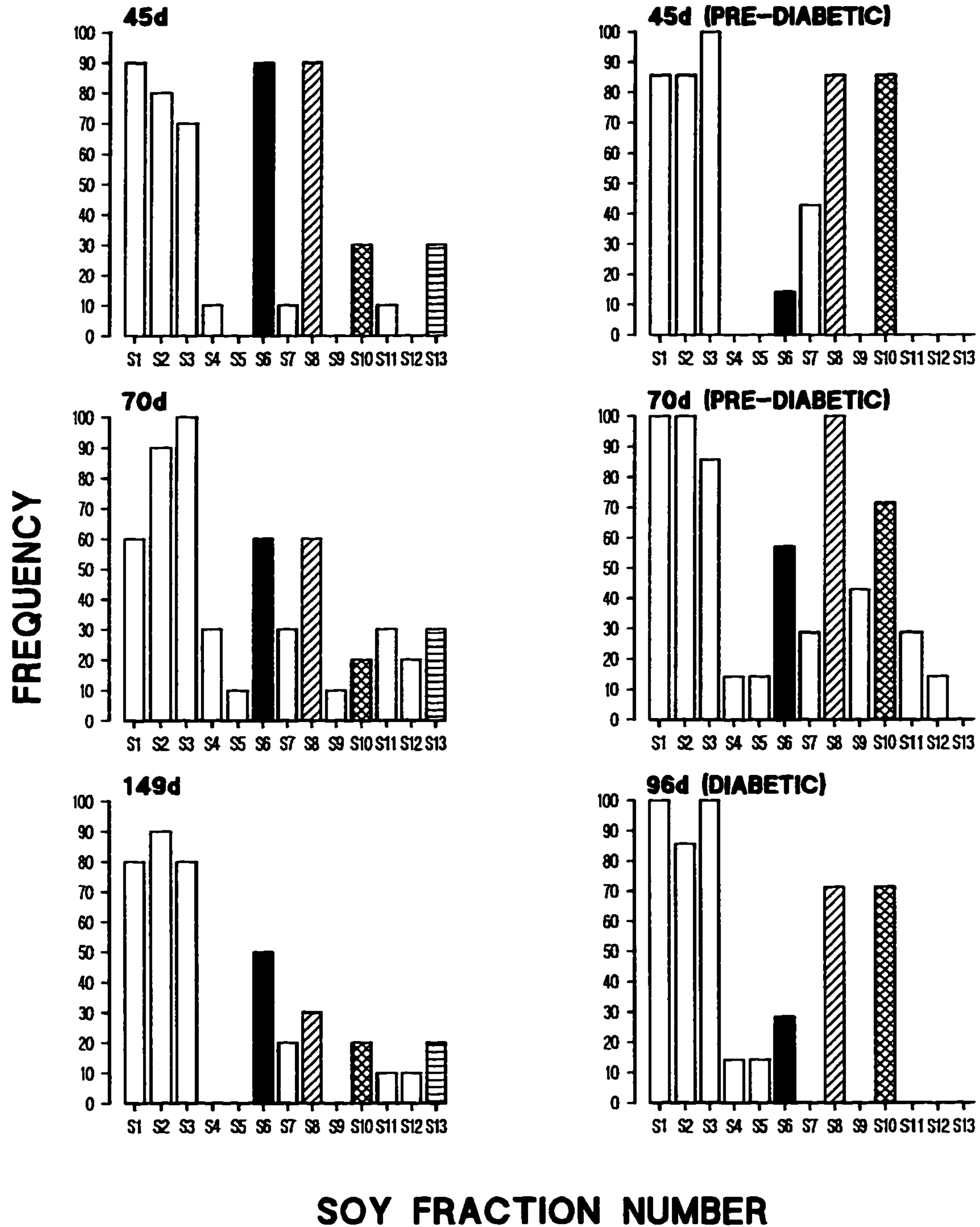

SPI DIET
ASYMPTOMATIC
PRE-DIABETIC OR DIABETIC
45d
45d (PRE-DIABETIC)
70d
70d (PRE-DIABETIC)
149d
96d (DIABETIC)
FREQUENCY
S1 S2 S3 S4 S5 S6 S7 S8 S9 S10 S11 S12 S13
SOY FRACTION NUMBER

Figure 9. Frequency of IgG antibody binding to soy fractions in the SPI extract in asymptomatic or diabetes-prone and diabetic BB rats fed Prosobee powder soy-based infant formula at various ages. Prosobee powder soy-based infant formula proteins were fed to 17 BBdp rats and the outcome of diabetes was measured at the end of the study at 149 d. Based on diabetes outcome, animals were divided into two groups of asymptomatic (n=11) and pre-diabetic or diabetic (n=6). IgG antibodies were measured against the blood samples from these two groups of animals. SPI extract was the antigen and immunoreactive bands were visualized by DAB staining. 96 d is the mean age at diagnosis of diabetes in animals that developed diabetes

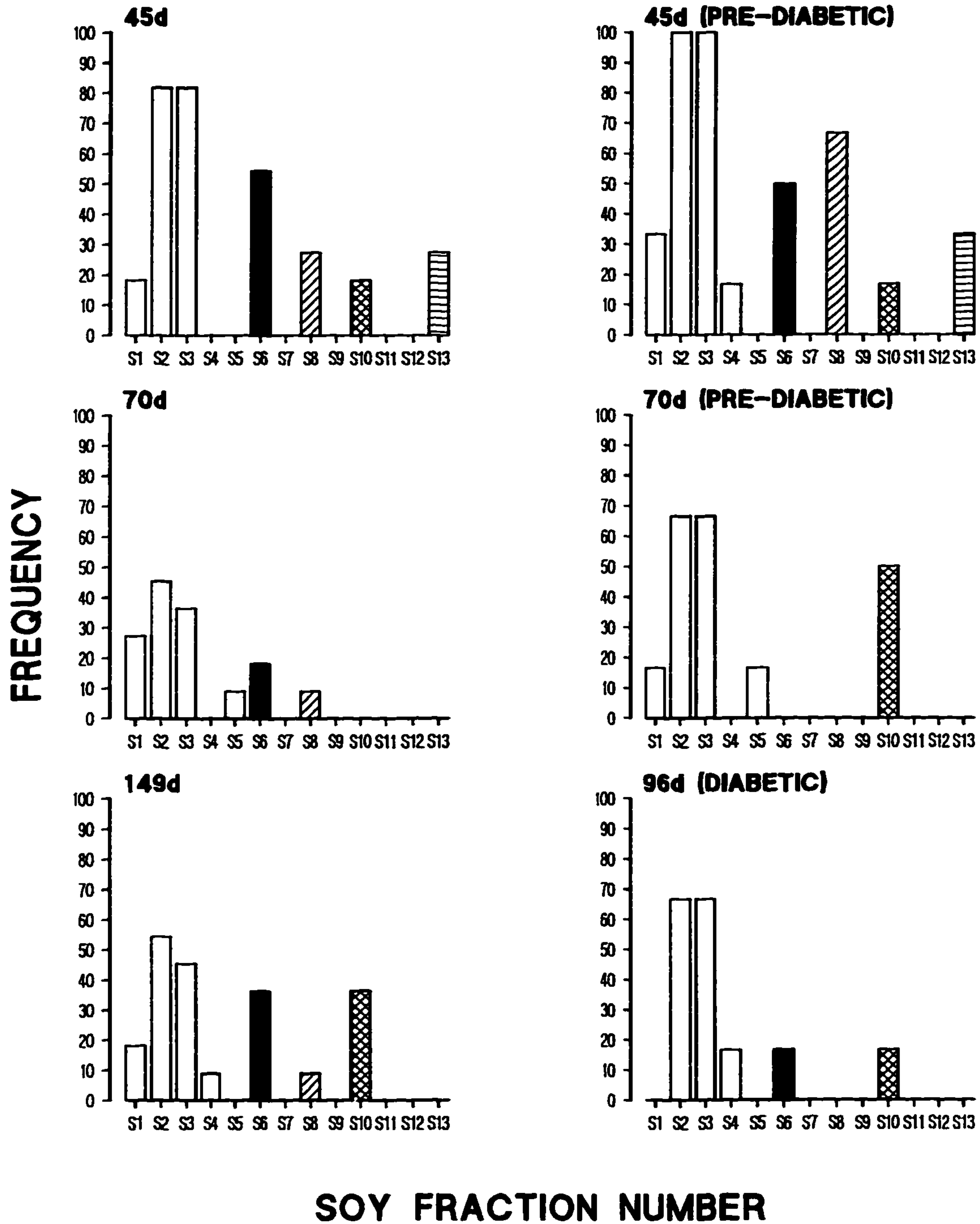

INFANT POWDER SOY FORMULA
ASYMPTOMATIC
PRE-DIABETIC OR DIABETIC
45d
45d (PRE-DIABETIC)
70d
70d (PRE-DIABETIC)
149d
96d (DIABETIC)
FREQUENCY
0
10
20
30
40
50
60
70
80
90
100
S1 S2 S3 S4 S5 S6 S7 S8 S9 S10 S11 S12 S13
SOY FRACTION NUMBER

Figure 10. Frequency of IgG antibody binding to soy fractions in the SPI extract in asymptomatic or diabetes-prone and diabetic BB rats fed Prosobee liquid soy-based infant formula at various ages. Prosobee liquid soy-based infant formula proteins were fed to 17 BBdp rats and the outcome of diabetes was measured at the end of the study at 149 d. Based on diabetes outcome, animals were divided into two groups of asymptomatic (n=13) and pre-diabetic or diabetic (n=3). IgG antibodies were measured against the blood samples from these two groups of animals. SPI extract was the antigen and immunoreactive bands were visualized by DAB staining. 96 d is the mean age at diagnosis of diabetes in animals that develop diabetes.

INFANT LIQUID SOY FORMULA

ASYMPTOMATIC **PRE-DIABETIC OR DIABETIC**

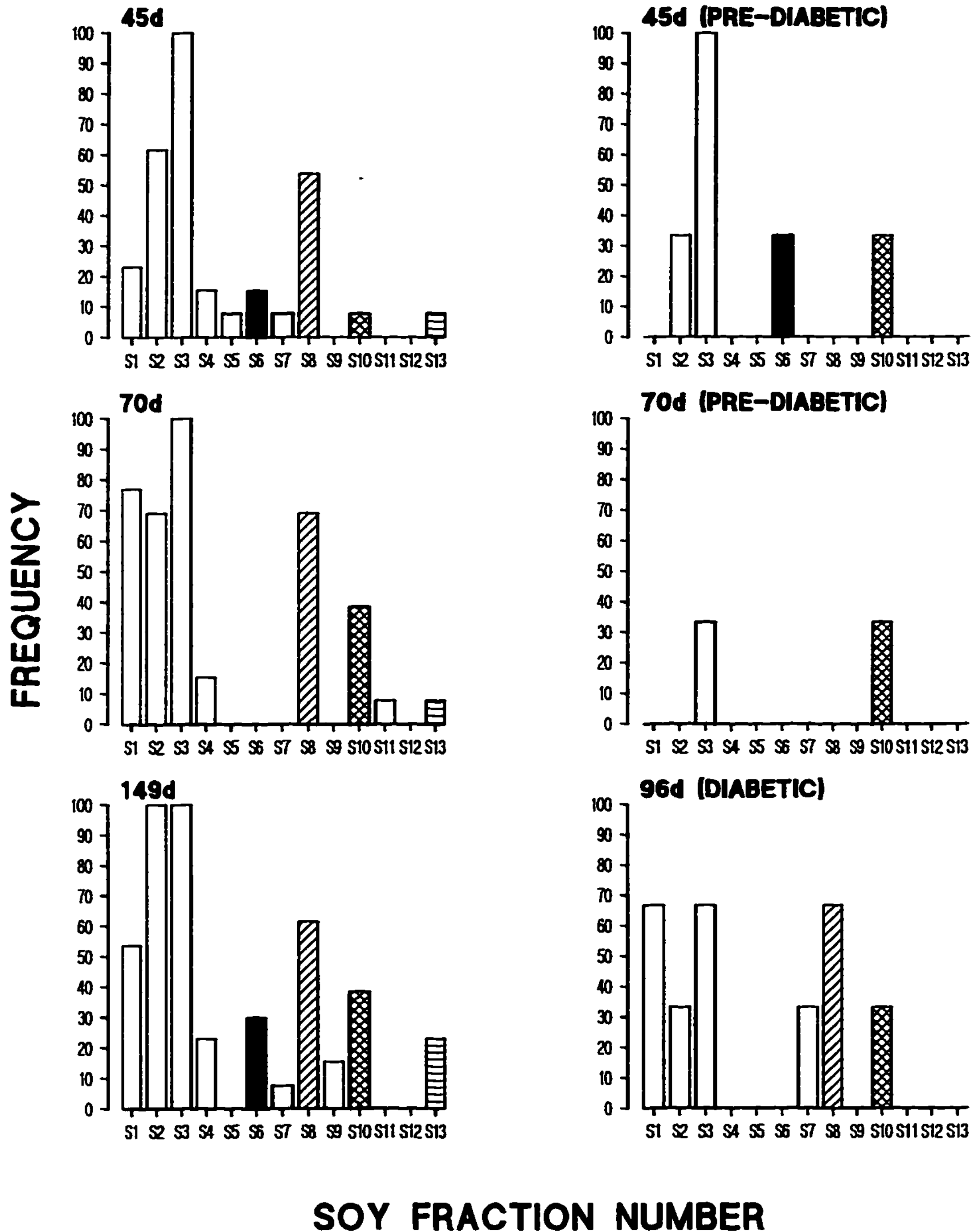
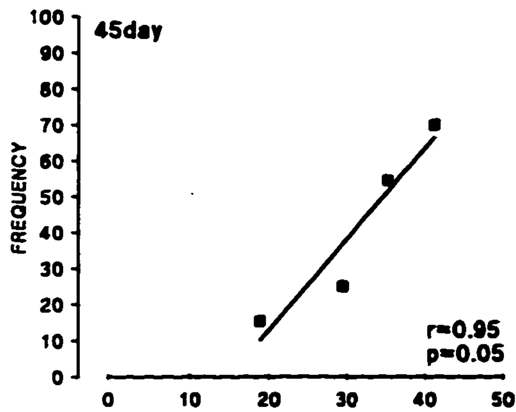
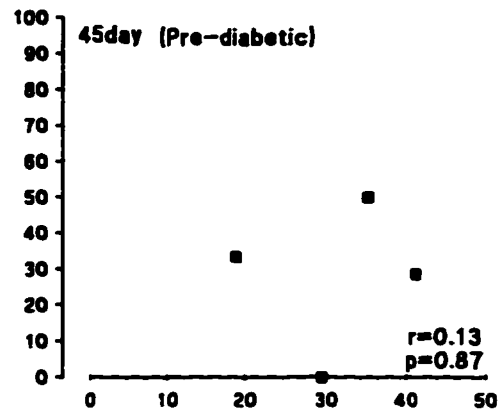


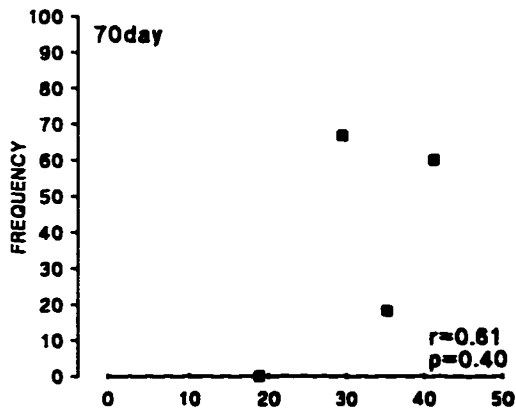
Figure 11. Correlation between various soy diets with different diabetes incidence and frequency of the S6 band at different ages. Animals were divided into two groups of asymptomatic and pre-diabetic or diabetic based on the diabetes outcome. Diets from lowest diabetes incidence to highest are Prosobee liquid soy-based infant formula (19%), hydrolysed soy protein isolate (29%), Prosobee powder soy-based infant formula (35%) and soy protein isolate (41%). 96 d is the mean age at diagnosis of diabetes in animals that develop diabetes.

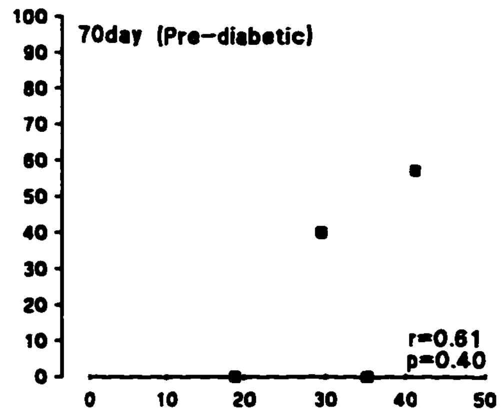
S6 BAND, IgG

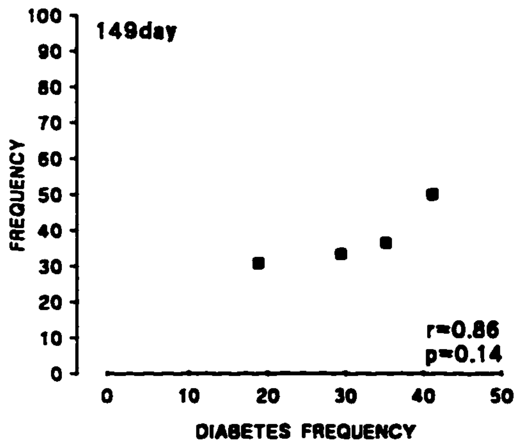
ASYMPTOMATIC   PRE-DIABETIC OR DIABETIC











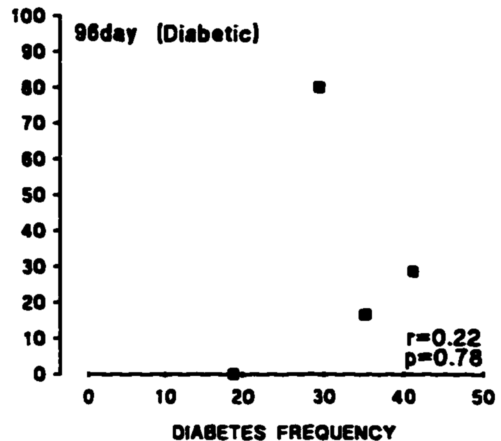
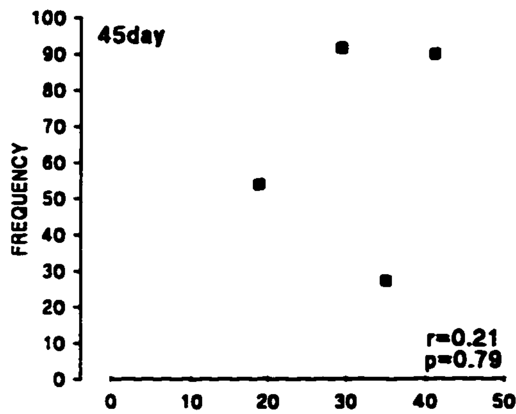
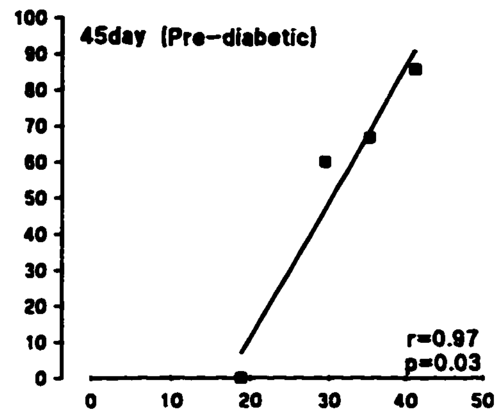


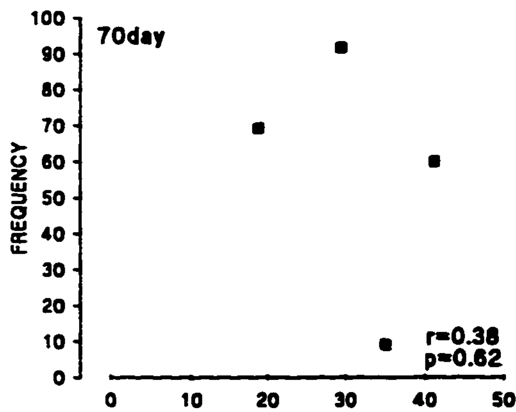
Figure 12. Correlation between various soy diets with different diabetes incidence and frequency of the S8 band at different ages. Animals were divided into two groups of asymptomatic and pre-diabetic or diabetic based on the diabetes outcome. Diets from lowest diabetes incidence to highest are Prosobee liquid soy-based infant formula (19%), hydrolysed soy protein isolate (29%), Prosobee powder soy-based infant formula (35%) and soy protein isolate (41%). 96 d is the mean age at diagnosis of diabetes in animals that develop diabetes.

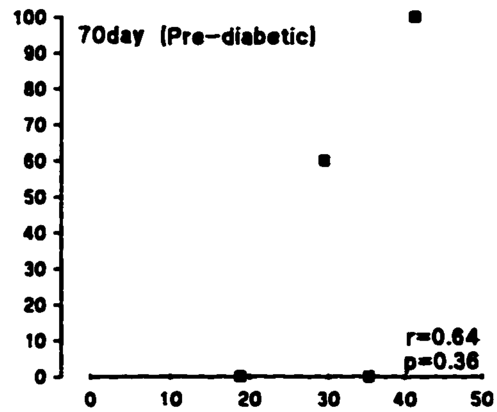
S8 BAND, IgG

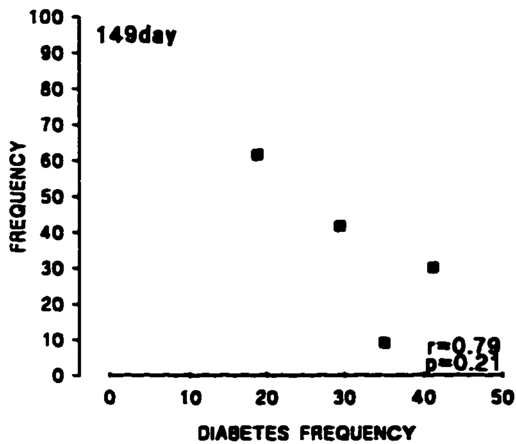
ASYMPTOMATIC   PRE-DIABETIC OR DIABETIC











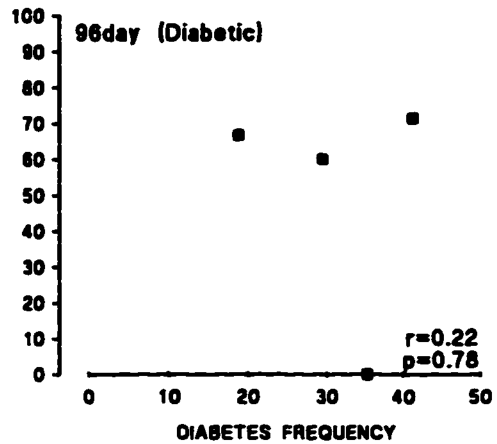
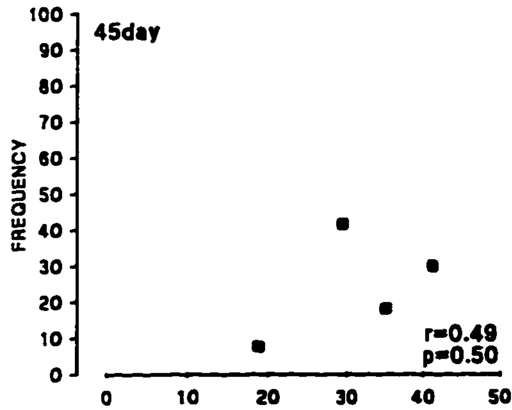
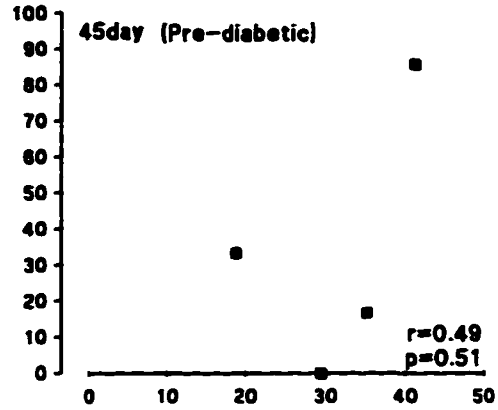


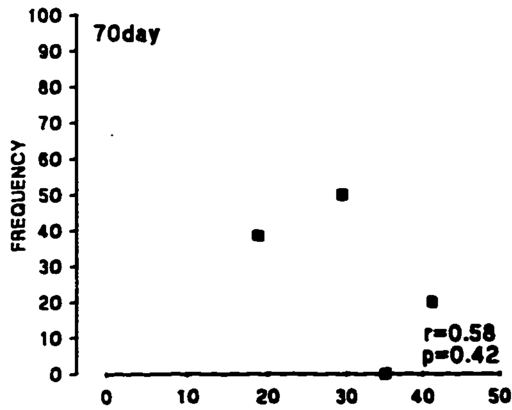
Figure 13. Correlation between various soy diets with different diabetes incidence and frequency of the S10 band at different ages. Animals were divided into two groups of asymptomatic and pre-diabetic or diabetic based on the diabetes outcome. Diets from lowest diabetes incidence to highest are Prosobee liquid soy-based infant formula (19%), hydrolysed soy protein isolate (29%), Prosobee powder soy-based infant formula (35%) and soy protein isolate (41%). 96 d is the mean age at diagnosis of diabetes in animals that develop diabetes.

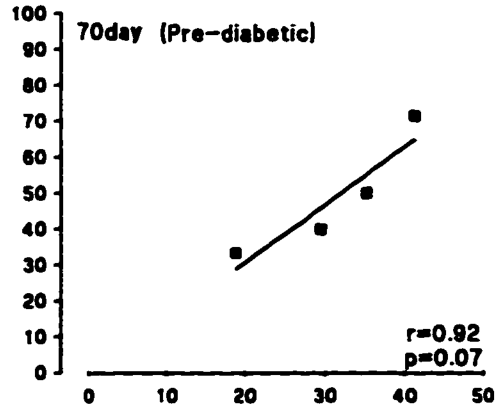
S10 BAND, IgG

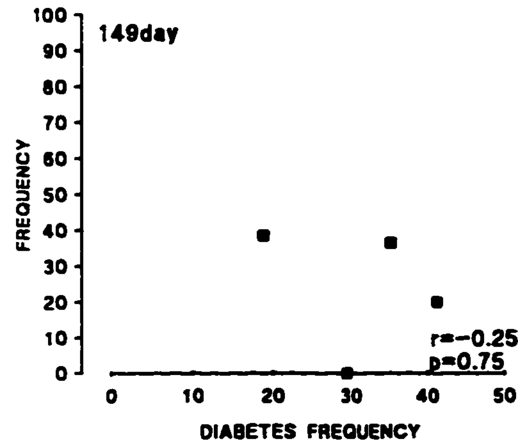
ASYMPTOMATIC   PRE-DIABETIC OR DIABETIC











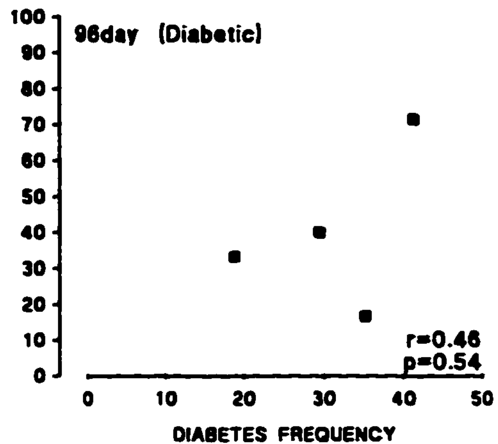
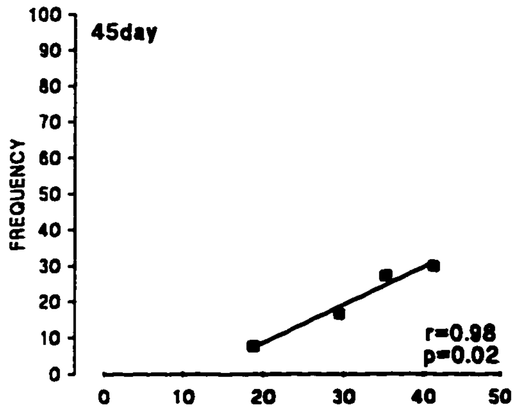
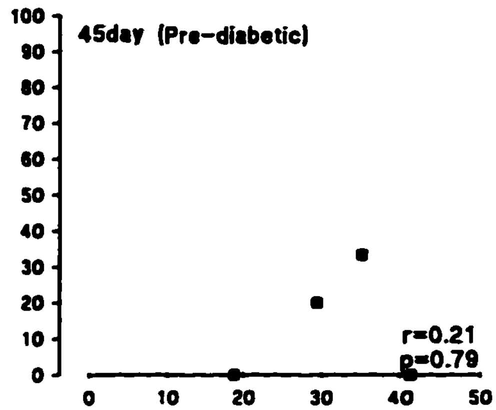


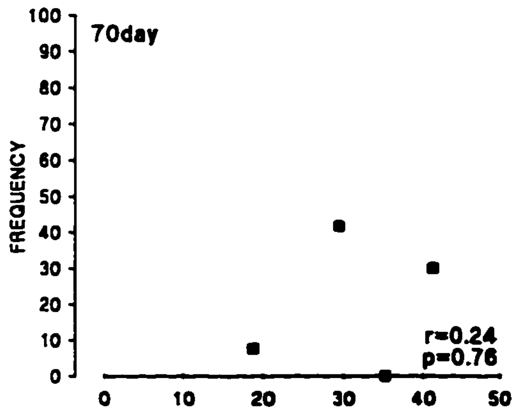
Figure 14. Correlation between various soy diets with different diabetes incidence and frequency of the S13 band at different ages. Animals were divided into two groups of asymptomatic and pre-diabetic or diabetic based on the diabetes outcome. Diets from lowest diabetes incidence to highest are Prosobee liquid soy-based infant formula (19%), hydrolysed soy protein isolate (29%), Prosobee powder soy-based infant formula (35%) and soy protein isolate (41%). 96 d is the mean age at diagnosis of diabetes in animals that develop diabetes.

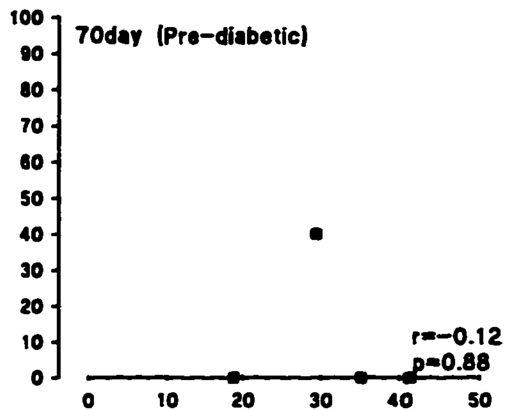
S13 BAND, IgG

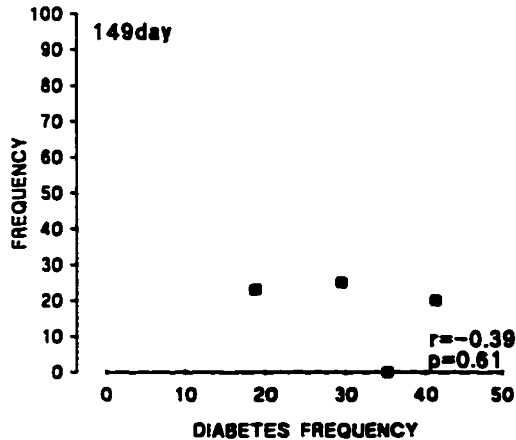
ASYMPTOMATIC   PRE-DIABETIC OR DIABETIC











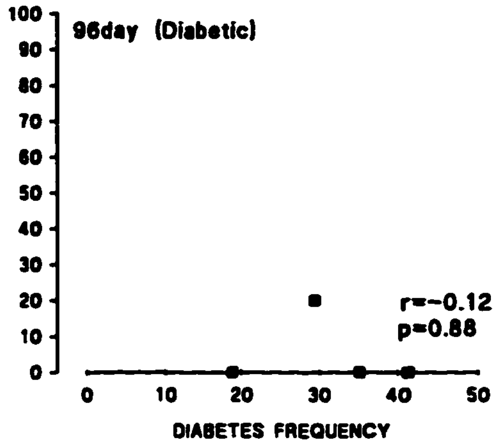
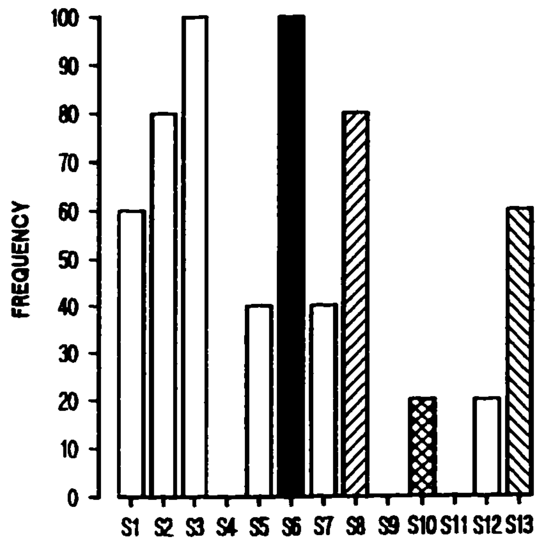


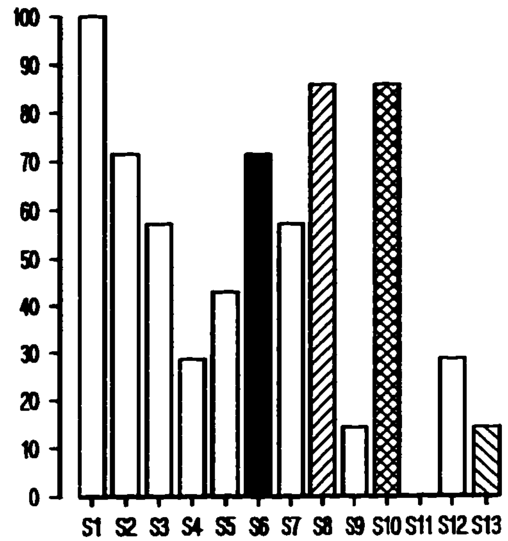
Figure 15. A. Frequency of IgG antibodies against the proteins extracted from soybean meal in two groups of volunteer controls (n=5) from Health Canada and newly diagnosed diabetic children (n=7) from CHEO.
B. Frequency of IgG antibodies against the proteins extracted from soy protein isolate (SPI) in two groups of volunteer controls (n=5) from Health Canada and newly diagnosed diabetic children (n=7) from CHEO.

A. SOY MEAL, CHEO (IgG)

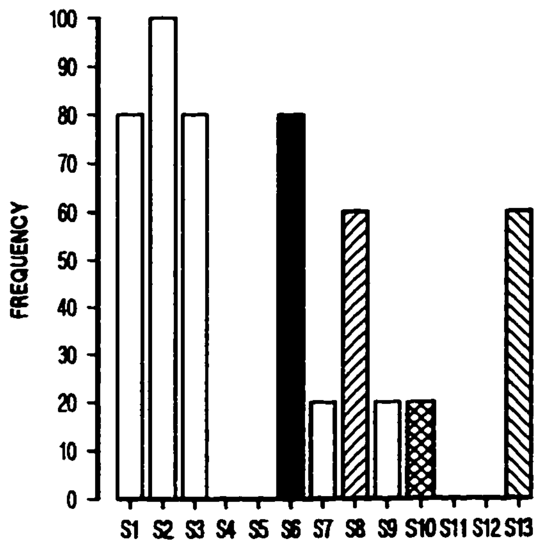
CONTROL



DIABETIC



B. SPI, CHEO (IgG)



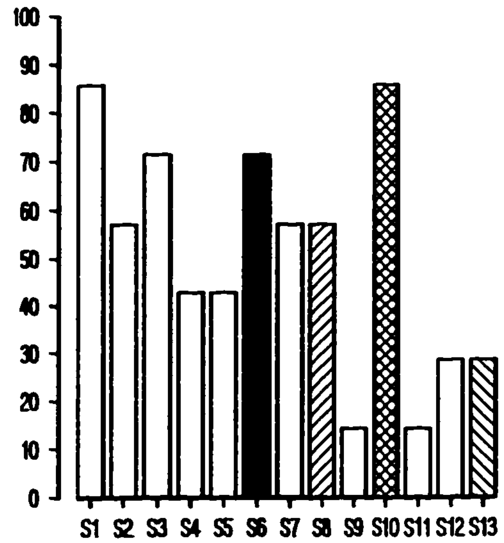
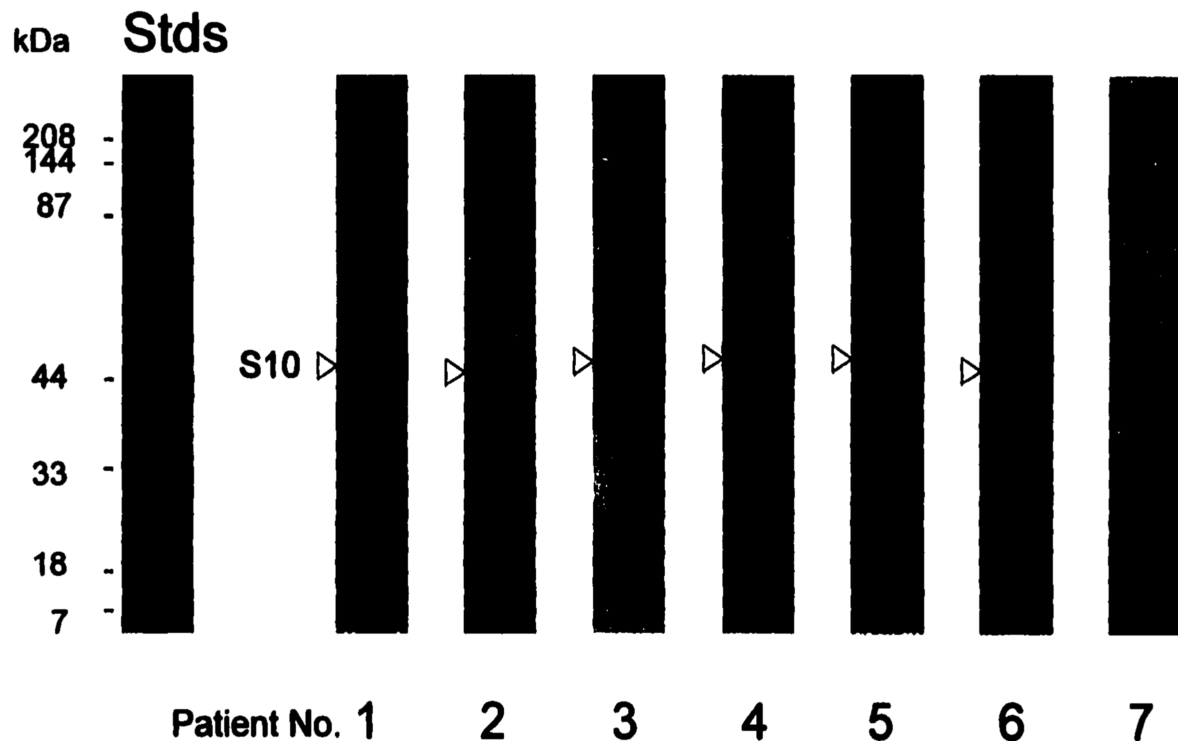


Figure 16. Western blots of soy protein isolate extracts using sera from newly diagnosed type I diabetic children (Ottawa study). Soy protein isolate extracts were separated on SDS-PAGE gel electrophoresis, followed by Western blotting. Serum samples from newly diagnosed type I diabetic children from CHEO at 1:1 dilution were used as the primary antibody. Goat anti-Human IgG-HRP at 1:100 dilution was the secondary antibody. Immunoreactive bands were visualized using diaminobenzidine (DAB) stain solution.

Newly Diagnosed Diabetic Children (CHEO)

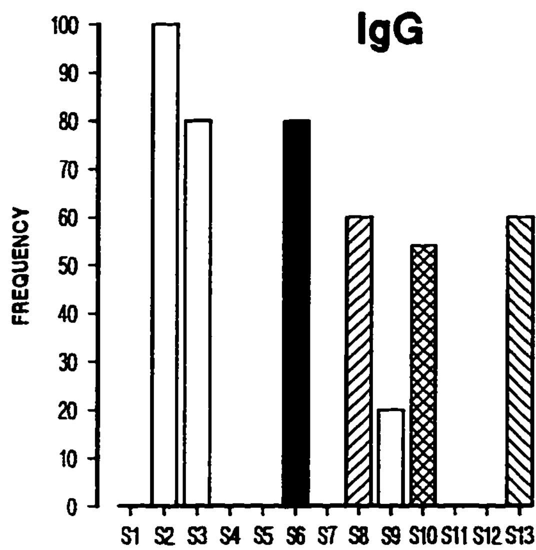


Soy Protein Isolate

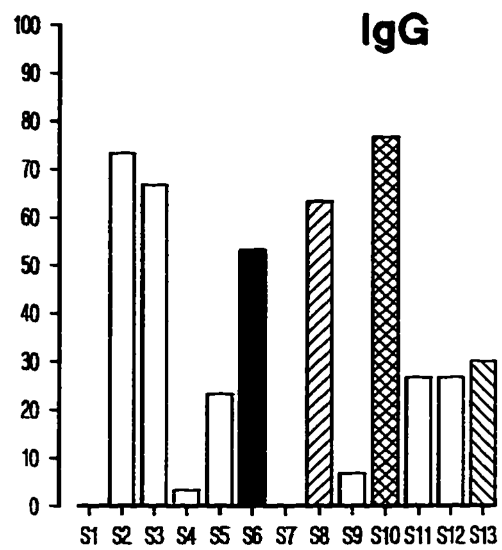
Figure 17. Frequency of IgG and IgA antibodies against proteins extracted from soy protein isolate (SPI) in two groups of age-matched controls (n=37) and newly diagnosed diabetic children (n=30) from Finland.

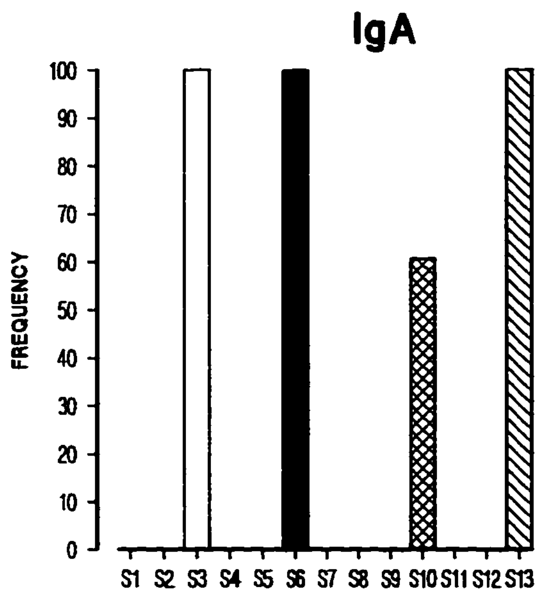
SPI EXTRACT, FINLAND

CONTROL



DIABETIC





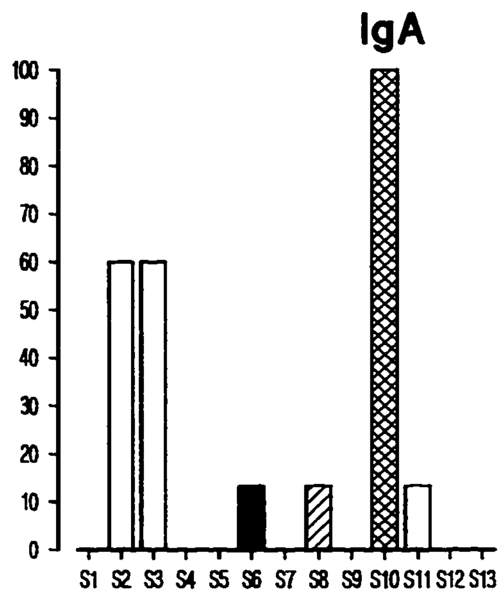
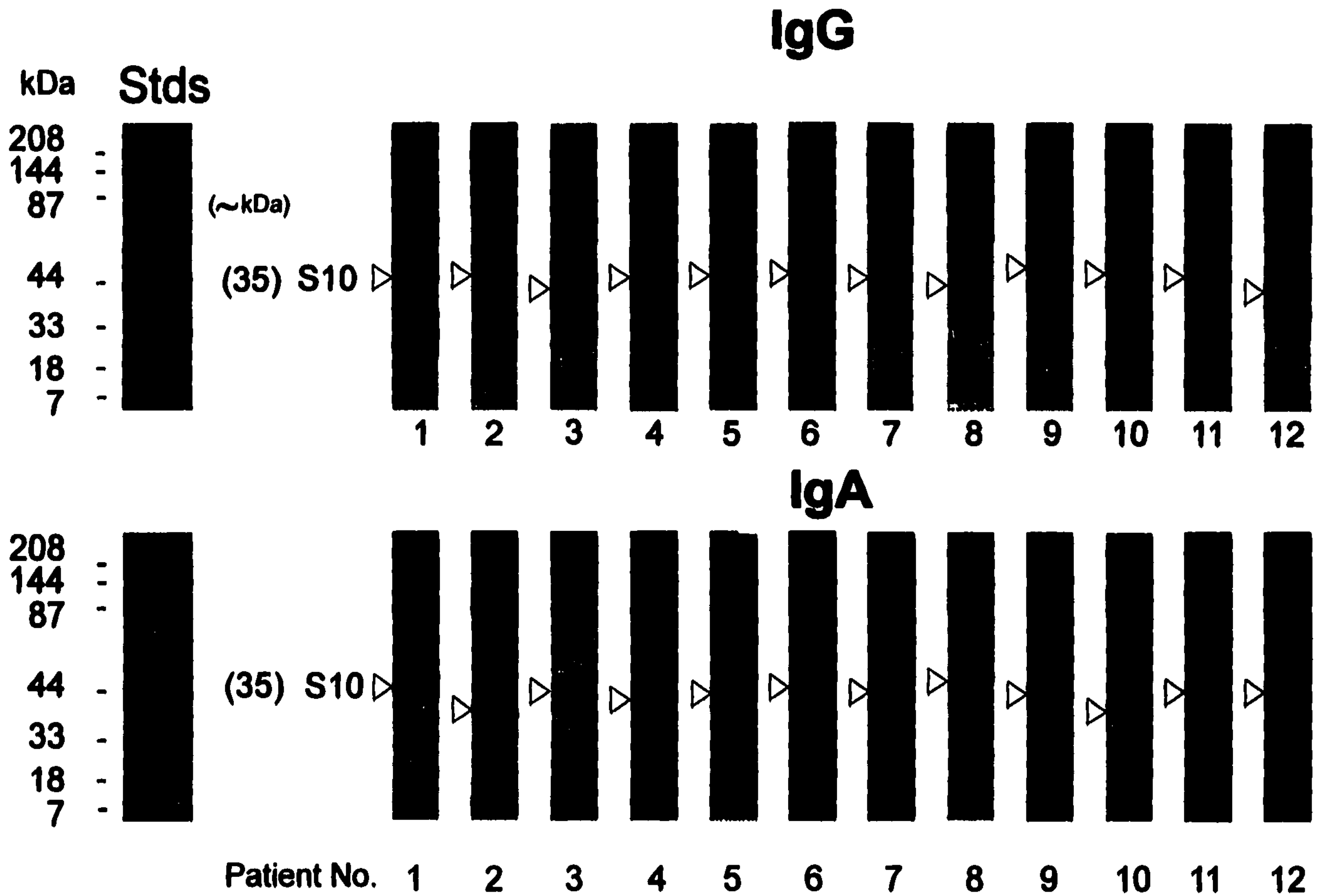


Figure 18. Selected Western blots of soy protein isolate protein extracts using sera from newly diagnosed type I diabetic children (Finland study). Soy protein isolate protein extracts were separated on SDS-PAGE gel electrophoresis, followed by Western blotting. Serum samples from newly diagnosed Finnish type I diabetic children at 1:1 dilution were used as the primary antibody. Goat anti-Human IgG-HRP and goat anti-Human IgA-HRP at 1:100 dilution were the secondary antibodies. Immunoreactive bands were visualized using diaminobenzidine (DAB) stain solution.



Newly Diagnosed Diabetic Children (Finland)
IgG
kDa
Stds
208
144
87
44
33
18
7
(~kDa)
(35) S10
1 2 3 4 5 6 7 8 9 10 11 12
IgA
208
144
87
44
33
18
7
(35) S10
Patient No. 1 2 3 4 5 6 7 8 9 10 11 12
Soy Protein Isolate

Figure 19. Early oral dosing with soy diabetogens. Survival curves showing incidence of diabetes in groups of BBdp rats (n=15 in each group) which were dosed orally with soybean meal in Pregestimil between days 4-7 and weaned at 23 d to soybean meal diet and those dosed orally with Pregestimil vehicle alone; weaned at 23 d to soybean meal diet. Another control group of animals was dosed orally between days 4-7 and weaned at 23 d to the protective HC (Hydrolysed Casein) diet.

EARLY ORAL DOSING WITH SOYMEAL

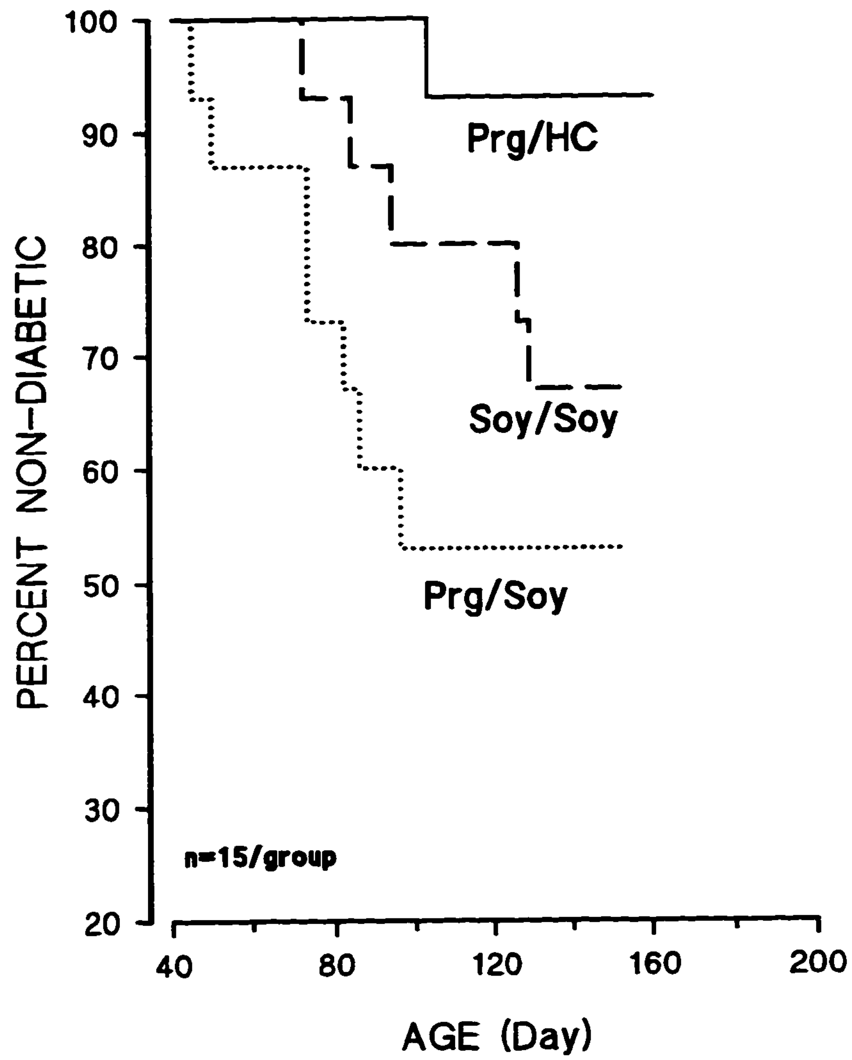
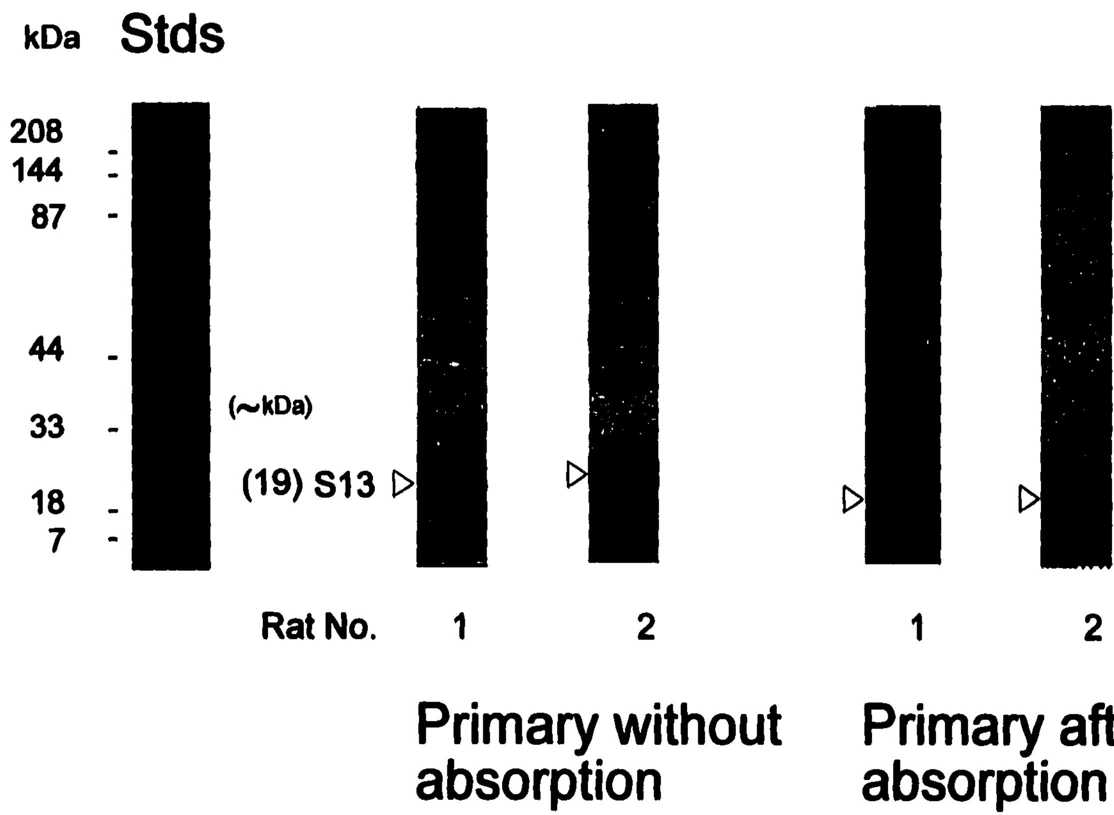


Figure 20. Western blots of soy protein isolate protein extracts using BB rat sera pre-absorbed with RIN cells. Soy protein isolate protein extracts were separated on SDS-PAGE gel electrophoresis, followed by Western blotting. Pre-absorbed non-diabetes prone (BBc) and diabetic untreated (BBd-U) sera at 1:1 dilution was the primary antibody and rabbit anti-rat IgG-HRP at 1:800 dilution was the secondary antibody. Immunoreactive bands were visualized using diaminobenzidine (DAB) stain solution.

Pre-absorbing Antibodies In BBc Rat Serum With RIN Cells



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PUBLICATIONS AND ABSTRACTS:

(1) Full Paper:

Kolb, H., Wörz-Pagenstert, U., Kleemann, R., Rothe, H., Rowsell, P., Rastegar, S., and Scott, F.W. (In Press 1996) Insulin therapy of prediabetes suppresses Th1 associated gene expression in BB rat pancreas. Autoimmunity.

(2) Abstract:

Scott, F.W., and Rastegar, S., A model system to assess links between foods and development of an autoimmune disease, juvenile diabetes. (Organization for Economic Corporation Development, OECD, workshop on Toxicological and Nutritional Testing of Novel Foods, March, 1997.)