ORAL TOLERANCE AND IMMUNE MECHANISMS IN FOOD-INDUCED DIABETES

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ABSTRACT

Diet controls ~80% of type-I (insulin-dependent) diabetes in the diabetes-prone BioBreeding (BBdp) rat. This study was designed to define the relationship among diet, the gut immune system and the pancreas. BB rats were fed either a diabetogenic NIH-07 (NIH) diet or the diabetes protective, hydrolysed casein (HC) diet. Bovine serum albumin (BSA), ovalbumin (OVA), sheep red blood cells (SRBC) and NIH were given by gavage daily for 5 days. Both BBdp and the diabetes resistant BBc rat when fed NIH became unresponsive in antibody production to NIH antigens. None of the other oral antigen treatments induced tolerance. In delayed-type hypersensitivity (DTH) reactions, footpad injection of NIH resulted in lower DTH reactions and less increase in popliteal lymph node weight when animals were fed NIH than HC. We conclude that oral tolerance, both cell-mediated and humoral, to diabetogenic antigens is inducible in both strains of BB rats. This required daily feeding unlike in other rat strains. The depressed DTH reaction in the animals fed NIH indicates no link between the systemic Th1 DTH reaction to NIH and the Th1 food-induced diabetogenesis. Neonatal intrathymic injection of autoclaved NIH did not affect diabetes incidence, suggesting systemic exposure to these food antigens was not protective. Feeding neonatal BBdp rats a diabetogenic diet between 4 and 7d of age significantly delayed diabetes and reduced incidence. This effect was seen with the NIH diet and its diabetogenic component, wheat gluten. We conclude that early exposure to food diabetogens is protective against food-induced diabetes, indicating a crucial link between the local gut immune system and autoimmunity against pancreatic β cells.

DEDICATION

This is dedicated to my father, Dr. Harry Rowsell, who first opened my eyes to the beauty of science.

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ABBREVIATIONS

ABTS	2,2'-Azino-di(3-ethyl- benzthiazoline-6- sulfonic acid; ELISA substrate
AIN-76	a standard defined semi-purified rodent diet
ANOVA	analysis of variance
BB	BioBreeding, the BioBreeding diabetic rat
BBc	partially inbred control diabetes resistant BioBreeding subline of rats (Ottawa)
BBdp	partially inbred diabetes-prone BioBreeding subline of rats (Ottawa)
BBdr	inbred diabetes resistant BioBreeding subline of rats (Worcester)
BSA	bovine serum albumin
d	day(s)
DTH	delayed type hypersensitivity reaction
ELISA	enzyme-linked immunosorbent assay
GLP-1	glucagon-like peptide 1
h	hour(s)
H&E	hematoxylin and eosin
HC	hydrolysed casein
IDDM	insulin dependent diabetes mellitus; Type I diabetes
IFN-γ	interferon gamma
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IL-2	interleukin 2
IL-4	interleukin 4
IL-5	interleukin 5
IL-10	interleukin 10
MHC	major histocompatability complex
NAD	nicotinamide adenine dinucleotide
NIH	NIH-07, an open formula rodent diet based mostly on plant proteins (% ingredients known)
NOD	nonobese diabetic mouse
O.D.	optical density
OVA	ovalbumin
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RT6	a rat T cell surface marker characteristic of mature T cells
S.E.	standard error
SRBC	sheep red blood cells
TGF-β	transforming growth factor beta
Th1	cytokines of the T-cell helper type 1
Th2	cytokines of the T-cell helper type 2
TNF-β	tumour necrosis factor beta
WG	wheat gluten

INTRODUCTION

IDDM is an Autoimmune Disease

Juvenile onset or insulin-dependent diabetes mellitus (IDDM) is a disease in which the insulin secreting β-cells of the pancreatic islets of Langerhans are destroyed by the immune system, leaving the patient forever dependent on exogenous insulin. Such treatment keeps the diabetic individual alive but with markediy enhanced risk for many complications such as diabetic nephropathy, neuropathy, vascular disease, blindness and early mortality.

Diabetes is a T cell mediated, organ specific autoimmune disease (Bach, 1994; Reich, Janeway, Visintin and Sherwin, 1993) in which the islets become infiltrated with mononuclear cells, a process termed insulitis. After the destructive process has run its course over several years, most of the insulin-secreting β cells in the islets have been destroyed. Generally, patients have circulating antibodies to proteins associated with the β-cells. Although the role of autoantibodies in organ specific autoimmune disease is uncertain, anti-glutamic acid decarboxylase and anti-insulin antibodies are common in IDDM, and are used clinically to judge the status of siblings at risk of developing diabetes. At least a dozen other autoantigens have been identified (Atkinson and Maclaren, 1993; Nepom, 1995). Higher levels of islet-cell autoantibodies, especially to glutamic acid decarboxylase, are associated with a higher risk of developing IDDM.

Genetic Susceptibility Modified by the Environment

Studies of families with one or more affected individuals have shown that

there is a strong genetic component to IDDM. The major histocompatibility complex (MHC), which is a crucial element in antigen recognition and regulation of the immune system, has the strongest linkage to the disease (Davies, Kawaguchi, Bennett, et al, 1994). Many normal individuals carry the same MHC alleles without suffering from the disease. Studies of identical twins show concordance in only one third of the pairs while 12% of dizygotic twins are concordant (Rowe and Leslie, 1995). This lack of concordance is a strong indication that there are factors besides genetic susceptibility involved and has led to the investigation of the role of environmental factors in the etiology of the disease.

The incidence of diabetes has been rising in Western countries in the past few decades (Bingley and Gale,1989) and it affects about 0.3% of people in the West (Rossini, Greiner, Friedman and Mordes,1993). The extent and rapidity of the increase in incidence is probably due to environmental factors. The increased survival of individuals with diabetes in the population and the better outcome in diabetic pregnancies is insufficient to explain the increase in incidence. In only one in eight new cases is there an affected relative. Diabetes incidence varies among various countries with Finland (42.8/100,000 person·year) having the highest incidence in the world and Japan (0.6/100,000 person·year) the lowest (Lévy-Marchal, Patterson, Green and the EURODIAB ACE Study Group, 1995). Studies of immigrant populations suggest that these

people tend to display the incidence of diabetes of the adopted country, again supporting a role for the environment in diabetogenesis.

Since at least the turn of this century, it has been suggested that infectious agents might affect diabetes expression. However, there is no conclusive evidence that viruses explain most diabetes cases nor the current rise in incidence. Given the current levels of immunization in children, it seems unlikely that childhood viral diseases are more common today than in the past. Recently, the pancreata of patients who died of acute diabetes, and the peripheral blood of recent onset IDDM patients, have been probed for viral nucleic acid sequences using PCR. No evidence was found of an association between diabetes and the presence of candidate viral sequences (Foy, Quirke, Williams et al, 1995, Foy, Quirke, Lewis et al, 1995). The case for a viral etiology for IDDM is decidedly unproven and recent studies suggest that diabetogenic agents in the diet are major environmental cofactors (Scott, Cui, and Rowsell, 1994).

Animal Models

There are two major rodent models of spontaneous IDDM, the non-obese diabetic (NOD) mouse (Kikutani and Makino, 1992) and the diabetes-prone BioBreeding (BBdp) rat (Nakhooda, Like, Chappel, Murray and Marliss, 1977; Crisa, Mordes and Rossini, 1992). Both these rodent models share many features with the human disease. The most important common feature is that

the disease in all three species is autoimmune in nature. Autoreactive islet specific T cell clones have been isolated from all three species (Giordano, 1992; Bendelac, Carnaud, Boitard and Bach, 1987; Ellerman and Like, 1995) and inheritance of certain alleles in the major histocompatibility complex is an important risk factor for diabetes susceptibility in each species (Castaño and Eisenbarth, 1990). The disease is polygenic in nature, with multiple susceptibility loci mapped in the human, the mouse and the rat (Davies et al 1994; Jacob, Pettersson, Wilson et al, 1992; Serreze and Leiter, 1994). Each of the animal models has been bred to establish a diabetes resistant subline.

The BB rat was discovered in 1974 near Ottawa (Nakhooda et al.,1977; Chappel and Chappel, 1983). These animals spontaneously develop diabetes during puberty and adolescence, between 60 and 120 days of age, with a peak incidence near 90 days of age. Diabetes occurs equally in males and females. In the original Ottawa colony, currently housed at the Health Protection Branch of Health Canada, 50 to 70 percent of animals develop diabetes with few animals becoming diabetic at ages greater than 120 days. Insulitis is seen in virtually all diabetic animals, approximately 90-100%, and is common in non-diabetic animals (~70%). However, it is not a near universal feature as it is in the NOD mouse. The BB rat, like human patients who sometimes display polyendocrine autoimmune disorders, frequently develops spontaneous thyroiditis, another autoimmune disorder, although it does not progress to

hypothyroidism (Whalen, Rossini, Mordes and Greiner, 1995).

The BB rat displays a lymphopenia which does not appear in the human nor in the mouse model (Jackson, Rassi, Crump et al, 1981). Mature T cells displaying the CD5 antigen are reduced in the peripheral blood, in the spleen and in lymph nodes, possibly as a result of a thymic defect which it shares with the coisogenic subline of diabetes resistant (BBdr[1]) rats (Doukas, Mordes, Swymer et al, 1994). The $CD4^+$ helper/inducer T cell subset is reduced (Poussier, Nakhooda, Falk et al, 1982) and the $CD8^+$ cytotoxic/suppressor T cell subset is almost absent (Woda, Like, Padden and McFadden, 1986). There is a defect in development marked by a reduced expression of the differentiation marker RT6 on T cells, a glycophosphatidylinositol anchored surface protein with NAD glycohydrolase activity (Takada, Iida and Moss, 1994).

The characteristic lesion in IDDM, insulitis, an intense mononuclear infiltration within and around the islets of Langerhans, begins around puberty (50-60d) in the BB rat. MHC class I hyperexpression is seen in the islets well before disease is clinically evident and before "classic" light-microscopy-defined infiltration of mononuclear cells (Issa-Chergui et al., 1988; Li, Scott, Park and Yoon, 1995). These and many other similarities to the human disease meant the

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1 Each colony of BB rats is referred to by an abbreviation indicating its origin as in BB/Wor for the colony in Worcester, Massachusetts. The original colony in Ottawa is called simply BB. Inbred sublines are called BBdp and BBdr for diabetes prone and diabetes resistant respectively. The Ottawa colony is not completely inbred so its diabetes resistant line is designated BBc instead of BBdr.

availability of this model of spontaneous diabetes was a breakthrough in the study of diabetes pathogenesis (Rossini et al., 1993).

Diet and IDDM

Perhaps the most interesting and possibly the most important of the manipulations which prevent diabetes in susceptible rodents is dietary modification. In both the NOD mouse and the BBdp rat, diet can control the expression of the disease (Scott, 1994a; Scott, Cui and Rowsell, 1994). Diets based on diabetogen-free amino acid sources such as casein, hydrolysed casein or an amino acid mixture inhibit the appearance of the disease (Scott and Trick, 1983; Scott, Trick, Hynie et al, 1984; Elliott and Martin, 1984; Scott, Mongeau, Kardish et al, 1985; Scott, Elliott and Kolb, 1989). Other amino acid sources such as wheat gluten and soybean are highly diabetogenic (Scott, Sarwar and Cloutier, 1988; Hoorfar, Scott and Cloutier, 1991). Scott reports that delaying introduction of a diabetogenic diet as late as 50 days after birth still leads to diabetes, although with a delayed onset (Scott, 1994b). The idea that there is a unique time period in which the environmental diabetogenic influence operates has led to an examination of the effect of early diet on the human disease.

It has been found in some studies that a shorter period of breast-feeding is associated with a higher incidence of IDDM (Borch-Johnsen et al, 1984), while early, sustained breast-feeding is protective (Christy, Zachai-Christiansen, Kastrup and Nerup, 1984). This has led some workers to implicate early

exposure to cow milk (Savilahti, Åkerblom, Tainio and Koskimies, 1988). Bovine serum albumin (BSA) is thought by some to be an important trigger of anti-islet cell immune reactivity because of the shared homology of a region of BSA and an islet protein, p69 (Karjalainen, Martin, Knip et al, 1992). However, in the BBdp rat model of IDDM, BSA was not a factor in increasing diabetes incidence (Scott, 1994a). A diet containing 5% BSA in a hypoallergenic infant formula did not increase the level of diabetes in the NOD mouse model of IDDM (Scott and Marliss, 1991). More recent studies, including two meta-analyses (Gerstein, 1994; Norris and Scott, 1996), suggest that milk may be a weak, and highly variable diabetogen. Studies in the BB rat and NOD mouse show that long term exposure to dietary food diabetogens, particularly around puberty, is required (Scott, Norris and Kolb, 1996).

Diabetes incidence in the BB rat can be modified by the source of dietary protein (Scott and Trick, 1983; Scott et al, 1984; Scott et al, 1985; Elliott and Martin, 1984). Plant-derived proteins, especially wheat gluten and soybean meal, may account for most of the diabetogenicity of cereal-based diets (Scott, Sarwar and Cloutier, 1988; Hoorfar, Scott and Cloutier, 1991). These were identified by examining the incidence of diabetes when animals were fed semipurified diets based on individual components of NIH, a standard open formula rodent diet, which was originally associated with high diabetes incidence (Hoorfar, Scott and Cloutier, 1991). Other components of the diet, such as

fishmeal, did not result in high diabetes incidence, while skim milk powder gave a variable incidence (reviewed in Scott, 1994a and in Scott et al. 1996). This phenomenon, the association of high diabetes incidence with plant protein dietary source, has been confirmed in the NOD mouse (Hoorfar, Buschard and Dagnaes-Hansen, 1993). Modifications of fat composition did not alter the level of diabetes in the BB rat (Scott, 1994b; Issa-Chergui, Guttmann, Seemayer et al, 1988). Changes in the source of carbohydrate from starch to either sucrose, fructose or lactose did not change the incidence of diabetes. Hydrolysed lactalbumin diets led to a low incidence of diabetes as did diets based on whole lactalbumin (Scott, 1988). Rapeseed meal, peanut meal, fish meal and kidney beans have been tested as the sole source of proteins in the diet and all have been found to result in a low incidence of diabetes in the BB rat (Scott, Sarwar and Cloutier, 1988). BBdp rats fed a hydrolysed casein-based diet (HC) have a diabetes incidence of $\leq$15% but when fed open formula cereal based diets such as NIH-07 or Purina 5001, the diabetes incidence is around 50-70%. These results show that the protective effect of feeding a diabetogen-free diet on the diabetogenic process must happen early in the life of the animal, likely around puberty (Scott and Marliss, 1991).

How diet affects the development of diabetes in animal models is not known. As the type of fats in the diet alters immune response and these have been shown to alter the course of autoimmune disease in other animal models

(Kelley, Ferretti, Izui and Strom, 1985), the effects of different fat sources on diabetogenesis have been studied. The natural history of diabetes in BBdp rats fed a non-diabetogenic (casein) diet was not influenced by changing the fat source (Issa-Chergui et al., 1988). The protective casein diet led to increased white blood cell (WBC) counts and increased thymus weight (Scott, Mongeau, Kardish et al, 1985). Thymus weight increase has not been substantiated in later studies but our laboratory has confirmed the increase in WBC in HC-fed compared with non-diabetic NIH fed animals (Scott, submitted). Decreases in WBC also have been seen in prediabetic patients (Drell and Notkins, 1987). A slight but significant increase in the $CD8^+$ T cells was seen in animals fed the protective diet while a similarly small but significant decrease in macrophage populations was seen in animals fed the protective diet (Scott, Hoorfar and Cloutier, 1990; Field, 1995). Functional tests in the same study did not show any difference in mitogen stimulated proliferation in lymphocytes from animals on the different diets. BBdp rats have higher IgM antibody levels to food proteins such as bovine serum albumin and β lactoglobin and higher IgG antibodies to gliadin than BBc rats (Scott, Cloutier, Souligny et al, 1989). The effect of diet on diabetes incidence may be related to this phenomenon.

A substantial effect of the protective HC diet is to counteract the hyperexpression of MHC class I molecules on the surface of the β cell, which occurs when BBdp rats are fed the diabetogenic NIH diet (Li, Scott, Park and

Yoon, 1995). A recent study examined parameters of the immune system of BBdp rats fed a protective purified casein-based diet and a diabetogenic nonpurified closed formula rodent diet (Field, 1995). This study reports a significantly lower number of macrophages in the spleens of BBdp rats fed the protective diet compared to those fed the diabetogenic diet. Total T cells in the mesenteric lymph nodes were significantly increased in animals fed the protective diet. Cytotoxicity by natural killer cells was lower in BBdp rats fed the protective diet. Measures of metabolic activity, which were interpreted as indicating the activation state of the cells, were lower in mesenteric lymph node cells from rats fed the protective diet. The effect of diet on immune measures indicates a slight decrease in activity in animals fed the protective diet. The greater activity of cells from the rats fed the diabetogenic diet, together with the hyperexpression of MHC class I molecules on the pancreatic β cells are indications of the immunological effect of diet on the diabetes process, but do not yet give us a mechanism to explain this effect. Changes in the mesenteric lymph node due to diet are of great interest as these changes would affect both islet cell reactive and islet cell protective lymphocytes derived from the gut.

The Pathogenesis of IDDM and its Alteration

Despite many years of searching, the details of the pathogenesis of IDDM remain unclear. It is difficult to predict which individual in the general population will develop diabetes and therefore studies of the preclinical phase are limited.

Because of this, and the many similarities to human IDDM, the BBdp rat and NOD mouse animal models have been used extensively to study the pathogenesis of this disease. Examination of the kind of cellular infiltrate which appears in the islets before hyperglycemia has given valuable information on how the disease develops. In the BB rat, well before disease is apparent, macrophages have invaded the islets (Hanenberg, Kolb-Bachofen, Kantwerk-Funke and Kolb, 1989). There were macrophages in the exocrine tissue, at the islet periphery and inside the islets. The main subsets of T lymphocytes, the CD4 expressing helper cell and the CD8 expressing cytotoxic/suppressor cell, were seen inside the islet and were also present at the periphery of the islet. In one of the few studies in humans, islets were also seen to be infiltrated with macrophages, B lymphocytes and $CD4^+$ T lymphocytes and the majority of cells were $CD8^+$ T cells (Hänninen, Jalkanen, Salmi et al, 1992). When infiltrating cells were examined by sequential biopsy, the pancreata of BB rats were observed to be first infiltrated with activated macrophages (Kolb-Bachofen et al, 1992), then by lymphocytes, including $CD4^+$ T-cells, natural killer (NK) cells and B lymphocytes (Walker, Bone, Cooke and Baird, 1988). Dendritic cells, the most stimulatory professional antigen presenting cells of the immune system (Schriever and Nadler, 1992), are present in the early infiltrate (Voorbij, Jeuken, Kabel et al, 1989). Other studies have shown that $CD8^+$ cells are not only present in the insulitis lesion but are essential for the development of diabetes,

in spite of the very low numbers of $CD8^+$ expressing T cells in the lymphopenic BB rat (Weringer and Like, 1988). It is likely that the inflammatory process in all three species, rat, mouse and human, is similar.

Over the last decade, it has become clear that immune reactions are controlled by the pattern of cytokines produced at the site of the reaction. T helper cells ($CD4^+$) can be divided into two broad subclasses, based on their cytokine secretion pattern: Th1 cells, which produce IL-2, IFN-γ, and TNF-β, and Th2 cells, which produce IL-4, IL-5, TGF-β and IL-10 (Rabinovitch, 1994). In many cases, autoimmune diseases have been shown to be driven by Th1 type cells. In the NOD mouse, IFN-γ production in the islets was found to be associated with diabetes (Rabinovitch, Suarez-Pinzon, Sorensen et al, 1995). $CD4^+$ cells reactive to the islet cell autoantigen glutamic acid decarboxylase were found to be IFN-γ secreting in the NOD mouse (Kaufman, Clare-Salzler, Tian et al, 1993). Leukocytes isolated from the pancreata of BBdp rats were found to express Th1 cytokines (Rabinovitch, Suarez-Pinzon, El-Sheikh et al, 1996). Type 1 diabetes is thought to be a Th1-driven process.

In simple terms, humoral immune reactions are driven by Th2 cells; cellular immunity is driven by Th1 cells (Romagnani, 1994). In humans, humoral immunity (antibody secretion) to glutamic acid decarboxylase is associated with a better clinical outlook than cellular immunity to glutamic acid decarboxylase (Harrison, Honeyman, DeAizpurua et al, 1993). Approaches which ameliorate

cell mediated autoimmune disease have been found to switch the cells at the lesion site from a Th1 profile to a Th2 profile: oral tolerance to insulin in the NOD mouse (Hancock, Polanski, Zhang et al, 1995); essential fatty acid deficiency in the NOD mouse (Benhamou, Mullen, Clare-Salzler et al, 1995); insulin immunization of IDDM in NOD mice (Muir, Peck, Clare-Salzier et al, 1995) and in protection of BBdp rats fed a non-diabetogenic diet (Scott, Cloutier, Kleemann et al, 1996).

An early event in the development of the insulitis lesion is the expression of MHC class I molecules (Issa-Chergui et al, 1988). Although it was thought originally that MHC class II molecule expression was a hallmark of pathology (Botazzo, Dean, McNally et al, 1985), it has become clear that hyperexpression of MHC class I molecules on the β-cells is more important in diabetogenesis (Foulis et al, 1987).

In general, MHC class I molecules present internally generated proteins as a subset of the peptides generated by the internal proteolytic machinery. MHC class II molecules present peptides generated through an endocytic pathway from proteins ingested by the cell. MHC class I molecules present peptides to the T-cell receptor (TCR) on cells bearing a CD8 molecule. MHC class II molecules present peptides to the TCR on cells bearing a CD4 molecule. A selective process occurs in the thymus, eliminating cells incapable of properly presenting antigens and those specific to self antigens. MHC class I expression

was reduced in BB X Buffalo hybrid rats fed diabetes-retardant diets (Issa-Chergui et al., 1988). More recently, it has been found that MHC I over-expression can be seen in islets of BBdp rats from the Ottawa colony as early as 25 days of age (Li, Scott, Park and Yoon, 1995). Feeding a low incidence, hydrolysed casein-based, diet resulted in low or absent expression of MHC I in the islets. A high incidence, mainly plant-based, diet resulted in MHC class I hyperexpression in the islets even when macrophages were depleted by treatment with silica (Li et al., 1995).

So we have a situation in which dendritic cells and macrophages are important in the early stages of IDDM in the BB rat but in which the essential elements of the attack are the $CD8^+$ and $CD4^+$ T cells. IDDM in the rat, mouse and human is a disease in which macrophages are the earliest detectable cellular infiltrate, followed by autoreactive T cells and β cell destruction (Foulis, McGill and Farquharson, 1991). Early MHC class I hyperexpression may make the β-cell the target of these mononuclear cells. This hyperexpression may be induced by interferon-α expression by the β-cell itself (Huang, Hultgren, Dybdal and Stewart, 1994; Huang, Yuan, Goddard et al, 1995). The initiating event is probably some damage, possibly diet-induced, to the β-cell. Regulation of the infiltrating cells by a switch from a Th1 cytokine pattern to a Th2 pattern can abrogate the destructive cascade. (Scott, Cloutier, Kleemann et al, 1996).

Tolerance

Autoimmunity is a failure of tolerance mechanisms. While these are not fully understood, even after a century of study, it is clear that several elements are involved including control by both central and peripheral mechanisms. The elimination of self-reactive T-cells in the thymus during thymocyte development is the main mechanism of central tolerance. Peripheral tolerance is less well understood. However, self-reactive lymphocytes can be eliminated or become anergized (functionally unresponsive) in the periphery.

Tolerance can also be induced. Neonatal tolerance occurs when very young animals are exposed to cells from a donor animal. They will then accept organs from the donor later in life. Another form of immune tolerance is achieved by injecting antigens into the thymus of the animal (Tisch, Yang, Singer et al, 1993). Soluble protein injected in high concentration into the blood stream or complexed with immunoglobulins results in immune tolerance (Gammon and Sercarz, 1989). An important process which is attracting attention for its potential clinical applications is the phenomenon of oral tolerance. When an animal is fed an antigen, it generally displays reduced humoral and cell mediated activity towards that antigen (Mowat, 1987). This is thought to occur because of the microenvironment, the pattern of cytokine secretion and the antigen presenting cells, on which the antigen is first encountered (Weiner, Friedman, Miller et al, 1994).

The questions addressed in this study were:

i) Can oral tolerance be induced in the BB rat? Does it differ between the BBdp and the BBc rat and what bearing does this have on the development of diabetes?

ii) Can administration of food diabetogenic antigens either at different sites or at different times modify diabetes outcome?

iii) Is there an effect of diabetogenic versus non-diabetogenic diets on measures of immune reactivity in diabetes-prone BB rats?

Approaches used in the Current Studies

The mechanisms by which diet influences the development of diabetes have not been defined. Attention has been focused on the early period of life by the recent controversies over cow milk protein as a trigger of diabetes (Atkinson, Bowman, Kao et al, 1993 ; Dosch, Karjalainen and VanderMeulen, 1994; Atkinson, Kao and Maclaren, 1994). We have therefore addressed the question of exposure early in life to defined diets with widely different diabetogenicity (HC vs. NIH) (Hoorfar, Scott and Cloutier, 1991) in a well studied spontaneous animal model of diabetes, the BB rat.

We hypothesize that food diabetogens present in the NIH diet, but absent from the HC diet, induce diabetes via interactions with the gut immune system. The studies discussed in this paper were designed to investigate the relationship between age, exposure to antigens in the gut or other sites and

development of diabetes in the BB rat.

As the phenomenon of oral tolerance is known to influence immune reactions at remote sites within the body, and the first major encounter of food with the immune system occurs in the gut, it was decided to investigate whether tolerance could be induced in the young BB rat, aged 35 days, via the oral route. Any difference between the diabetes prone BBdp and the control BBc rat in ability to develop oral tolerance could indicate the nature of the food-diabetogens and possible sites of interactions between diet and genetic susceptibility. It is possible that these food components may affect directly the target β cells, or the effect could be on elements of the immune system, or both immune and target cells may be affected.

To approach these questions, different routes of exposure to these diabetogenic antigens in food were examined. One of the questions posed was whether exposure to these antigens in the neonatal thymus, where immunocytes are "educated", could reduce the incidence of diabetes in the BBdp. Presentation of these dietary antigens might then cause β cell reactive immunocytes to be eliminated, either physically or functionally (anergized). This would be strong evidence that the immune system recognized an element of the diet as similar to β cell antigens, a form of molecular mimicry (Nickerson, Luthra and David, 1991). As well, because the gut immune system develops in part separately from the thymus (Poussier and Julius, 1994), a potential exists to

influence its development by early exposure to diabetogenic dietary components. So animals were injected intrathymically at 24 hours of age with the diabetogenic diet. The role of the neonatal gut was investigated by hand feeding diabetogenic diet to suckling rats at 4-7 days of age.

Previous work in this laboratory has demonstrated a reproducible inhibition of diabetes expression in the BB rat by feeding diabetogen-free diets. Characterization of the elements in the diabetogenic NIH diet which enhance the development of diabetes is being investigated and is not the subject of this thesis. The diabetogenic components of the diet have been partially characterized and they are probably low molecular weight, glutenins from wheat and non-ethanol extractable, non-trypsin-inhibitor soy proteins (Scott et al., in preparation).

The studies reported here focus on the effects of using different routes of exposure to the diabetogenic dietary antigens on the immune system of the BB rat at different ages. Determining if a specific route of exposure leads to an elevated response or leads to tolerance would help to characterize the behaviour of the rat immune system to diabetogens in food. Any correlation with diabetes onset or incidence would be of interest in describing the mechanism of diet-induced IDDM. This will aid in understanding the pathogenesis of diabetes development in humans. It is to be hoped that preventive measures can be derived from this knowledge.

MATERIALS AND METHODS

Experiments were carried out using BB rats to examine the mechanisms involved in food-induced diabetogenesis as follows: 1) the ability to establish oral tolerance in antibody and cell-mediated responses to soluble or complex antigens in the BBdp and BBc rat fed the diabetogenic NIH diet or the protective HC diet at 35 days of age; 2) the ability to induce tolerance to the islet β cells by injection of diabetogenic NIH intrathymically, demonstrated by inhibition of diabetogenesis; 3) assess the immune reaction in a Th1 mediated process, the DTH reaction, of the two sublines of rat on the two diets to the NIH antigens; and 4) the effect that the exposure of the neonatal gut to diabetogenic diet or dietary components has on the rate and timing of diabetes incidence.

Animals

Diabetes prone BB rats (BBdp) and control BB rats (BBc) were obtained from the Animal Resources Division of Health Canada. BBc rats derive from an early subline in our colony which did not spontaneously develop diabetes. All animals were maintained under specific pathogen free conditions and are antibody-free with respect to Sendai virus, pneumonia virus of mice, rat carona virus/sialodacryoadenitis virus, Kilham rat virus, Toolan's H-1 virus, reovirus type 3 and mycoplasma pulmonis. Animals were weaned at 23 days of age, caged in banks of 30 wire bottom cages and given free access to food and water. Diets were fed in powdered form in open bowls. The animals were weighed at weekly

intervals between the ages of 30 days and 100 days. Animals were tested twice weekly for glucose in the urine after 60 days of age (Testape, Lily, Toronto, Ontario). Animals with 2+ or greater value were fasted overnight and blood glucose was measured the next morning in tail blood using glucose test strips and a glucometer. Animals were diagnosed as diabetic if fasting blood glucose was greater than 200mg/dL (11.1mM). Diabetic animals were killed within 24 hours of diagnosis by exsanguination under 5% isoflurane in oxygen anaesthesia. Pancreata were fixed in Bouin's solution for histology. Hematoxylin and eosin stained sections (H&E; 5μm) were read for insulitis and degree of islet damage under light microscopy (Hoorfar et al,1991). Briefly, the degree of mononuclear cell and macrophage infiltration of the islets (insulitis) was graded according to a scale of 1 to 5. Normal appearing islets were rated as 1; with 2 indicating mild infiltration; in 3, a majority of islets were infiltrated; in 4, most islets were heavily infiltrated; and 5 indicated endstage disease, with few, mostly small, islets, and with little inflammatory infiltrate remaining (examples are shown in Figure 1).

Diets

NIH

A standard rodent diet NIH-07 (NIH) was purchased in meal form from Ziegler Brothers (Gardners, Pa.) and stored at 4°C until use. NIH is an open formula, non-purified diet with protein sources provided mainly from plants

(82.5%). The components of the NIH-07 diet are: 5% dried skim milk; 10% fish meal; 12% soybean meal; 4% alfalfa meal; 3% corn gluten meal; 24.5% ground yellow shelled corn; 23% ground hard winter wheat; 10% wheat middlings; 2% brewer's dried yeast; 1.5% molasses; 2.5% soybean oil; 0.5% sodium chloride; 1.25% dicalcium phosphate; 0.5% ground limestone; and 0.25% premixes (Bieri, Stoewsand, Briggs et al, 1977).

Hydrolysed Casein

The hydrolysed casein diet consists of: 53.0% corn starch; 12.0% sucrose; 20.0% casein hydrolysate (Champlain Industries, Mississauga, Ont.); 5% corn oil; 5% alphacel; 3.5% AIN-76 mineral mix; 1.0% AIN-76A vitamin mix; 0.2% choline bitartrate; and 0.3% DL-methionine (Bieri, Stoewsand, Briggs et al, 1977; American Institute of Nutrition, 1980).

Wheat Gluten

Wheat gluten diets were made up with: 22.5% wheat gluten (ICN); 50.2% corn starch; 12.0% sucrose; 5.0% corn oil; 5% alphacel; 3.5% AIN-76 mineral mix (ICN); 1.0% AIN-76A vitamin mix (ICN Biochemicals, Cleveland, Ohio); 0.2% choline bitartrate; 0.02% DL-methionine; 0.5% L-lysine; and 0.08% L-threonine.

Serum Collection

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. All blood was put into serum separation tubes (Vacutainers, Becton Dickson, Rutherford, New Jersey), centrifuged for 10 minutes at 1000 X g and serum was stored at -20°C until used for analyses.

ELISA

Serum levels of IgG antibodies capable of binding selected food antigens or mixtures of food antigens were measured by ELISA using peroxidase linked secondary antibodies and ABTS (2,2'-Azino-di(3-ethyl- benzthiazoline-6-sulfonic acid) as substrate, read at 405 nm (Scott, Fausa, Ek and Brandtzaeg, 1984).

Plates were coated overnight at 4°C in pH 9.6, 0.1M carbonate buffer on Nunc-Immuno Maxisorp C96 flat bottom 96 well plates (Gibco/BRL, Burlington, Ontario). Plates were coated with NIH antigens as an aqueous extract at 200 µg/ml, equivalent to 50 µg protein. Plates were coated with sheep red blood cells (SRBC) by suspending them overnight at 4° C in phosphate buffered saline (PBS) at 1 X 10^8 cells / ml. Plates for the two soluble antigens, ovalbumin (OVA) and bovine serum albumin (BSA), were coated at 50 µg/ml. Plates were washed five times in PBS containing 0.5% Tween 20. Plates coated with SRBC or NIH were blocked with 1% gelatin in PBS for one hour at 37°C. Serum was diluted to 1:10, 1:100 and 1:1000. Negative control and positive control sera were used on the same plate with the sample sera and incubated for one hour at 37°C and washed. After one hour incubation with peroxidase linked rabbit

anti-rat IgG (ICN Biochemicals, Cleveland Ohio) at 37°C, the plates were washed and incubated with the substrate ABTS. As timing was crucial, all plates were incubated for exactly 15 minutes before reading. The plates were read at 405 nm with 490 nm as reference in a TiterTek Multiscan MCC Plate Reader (Flow Laboratories, Mississauga, Ont.).

Experimental Design

Experiment 1:The Ability to Establish Oral Tolerance in Antibody Responses to Soluble or Complex Antigens in the BB rat at 35 days of Age.

Protein sources in the diet can control the occurrence of diabetes in the BBdp rat. This experiment was designed to investigate the ability of the BBdp rat to establish oral tolerance, an important function of the gut immune system. The behaviour of the immune system of the BBdp rat in reaction to antigens presented via the gut was contrasted with that of the BBc rat. The influence of the diet was investigated by feeding the BBdp and the BBc rats the diabetogenic NIH-07 diet or a protective HC diet. Two soluble antigens, ovalbumin (OVA) and bovine serum albumin (BSA), were used to test the extent of oral tolerance established. A complex, T-cell dependent antigen, sheep red blood cells (SRBC), was used to see if the animals responded differently to this type of antigen. Finally, the complex mixture of antigens from a slurry of NIH-07 was tested in these animals. Saline was used as a control.

The results of the protocol to induce tolerance were used to characterize

changes in the antibody producing arm of the immune system. The protocol for establishment of oral tolerance was based on that of Troncone and Ferguson (1988) and Whitacre (Whitacre, Gienapp, Orosz and Bitar, 1991). The animals were fed daily doses of antigen by gavage for a period of five days. Serum antibody levels were compared between the week after the oral dosing and at two weeks after a challenge with antigen, or 28 days after the oral dosing regime.

At 35 days of age the animals, weighing between 120 and 130 g, were dosed daily by gavage with 1 ml of saline or 1 ml of saline containing 40 mg/ml of OVA (Sigma Chemical Co., Mississauga, Ont.) or of BSA (Sigma Chemical Co., Mississauga, Ont.) or 1 ml of saline containing 160mg of NIH (equivalent to 40 mg/ml of protein). The SRBC (Qualicum Scientific Ltd., Nepean, Ont.) were washed 3 times in Alsevers solution (Sigma Chemical Co., Mississauga, Ont.) and administered in 1ml of Alsevers solution at 4.5 X 10^9 cells per ml. The NIH was administered as a slurry. Blood was sampled before the systemic challenge by orbital bleed under light anaesthesia at 44 days of age. Animals were challenged s.c. with 25 μg of protein antigen (4.5 X 10^8 SRBC, NIH was autoclaved) in complete Freund's adjuvant at two sites, in the hind leg and in the neck with 100μl per site at 45 days. Seventy percent ethanol was used to cleanse the site before the injection. Blood was sampled again, by orbital bleed, after systemic challenge with the antigens, at 66 days.

Humoral response.

The two samples per animal, before and after systemic challenge, were used to measure changes in circulating antibodies after systemic challenge to the antigens used in the oral tolerance protocol. The experiment was designed to determine differences between the BBdp and the BBc rat in the ability to induce tolerance orally and to evaluate the possibility of a diet-induced effect by the diabetogenic NIH diet or the protective HC diet on oral tolerance.

Experiment 2: Intrathymic Injection of Dietary Antigens

If a component of the diet is triggering an immune response culminating in an immune attack on the β cells, it might be possible to induce tolerance in the animal to those dietary antigens and so prevent the diabetogenic effect of diet. It is generally accepted that most self antigens are presented to the developing T cells in the thymus. This process can be used to generate tolerance to foreign antigens by injecting cells or soluble antigens into the thymus in the neonate or later in life. Intrathymic injections of cells have been used by others to induce tolerance in animals before grafting foreign tissue (Posselt, Barker, Tomaszewski et al, 1990; Posselt, Odorico, Barker and Naji, 1992).

To investigate whether thymic exposure to dietary diabetogens could protect against diabetes, a second experiment was performed on day old BBdp rats. Day old pups were injected with autoclaved NIH-07 diet in both lobes of the thymus. Saline was used as a control. All animals were BBdp rats, fed a

diabetogenic NIH diet. Autoclaving NIH does not decrease the diabetogenicity of NIH (Scott et al,1994; Hoorfar et al 1993; Hoorfar et al 1991). Therefore, a direct injection of autoclaved NIH-07 into the neonatal rat should expose the thymic cells to dietary diabetogens. Pups were removed from the dams within 24 hours of the report of their birth. They were anesthetized, the upper thorax was opened using aseptic surgical techniques and the thymus was injected in each lobe with 50 µl of either saline or a slurry of ground, autoclaved NIH diet (20 minutes at 125°C) in saline (160 mg/ml). To identify them at weaning, animals receiving saline were tattooed in the left rear paw. After surgery the rats were returned to their dams. Recovery was monitored for one hour and any animals found to be in difficulty were killed. Dams were fed the diabetogenic NIH diet. At 23 days of age, animals were placed in individual wire bottom stainless steel cages and weaned onto the same NIH diet. Animals were assessed twice weekly for diabetes (polydypsia, polyuria, glucosuria, fasting hyperglycemia, as above) beginning at the age of 60 days.

Experiment 3: Popliteal Lymph Node Assay to Assess Th1 Cell Immune Reactivity to Diabetogenic Food Antigens

Experiment 3 was designed to investigate the immune reactivity to food diabetogenic antigens by systemic exposure to NIH antigens. The popliteal lymph nodes drain the lower leg and foot. Antigens injected into the footpad result in the activation of the popliteal lymph node cells and this results in an

increase in size. This is a classic functional test that measures T cell (Th1) reactivity (Askenase, 1992).

BBc and BBdp animals were fed diabetogenic NIH or protective HC diets. At 60 days of age the animals were lightly anaesthetized and injected with autoclaved NIH diet in Pregestimil® (0.5 mg/ml) in the left footpad and, as a control, with Pregestimil® in the right footpad. Pregestimil® is a hydrolysed casein based non-diabetogenic infant formula, consisting of: 13% hydrolysed casein, 62% carbohydrates (corn starch and corn syrup solids), modified coconut oil, supplemented with vitamins A, B_1, B_2, B_3, B_6, B_{12}, C, D, E, K , biotin, folic acid, α-pantothenic acid, the amino acids, L-tryptophan, L-cystine and L-tyrosine, as well as mineral supplements. This injection served as a control for the footpad injected with diabetogenic NIH, so we could compare the immune reactivity to the diabetogenic diet and a protective diet at a novel site. Footpads were cleaned with an iodine solution. The mixture was injected at 25 µl per footpad. Footpad thickness was measured before the injection for a zero time measurement. The time was noted and subsequent measures were performed at approximately the same time of day (± 1 h). Footpad thickness was measured with a micrometer (Mitutoyo, Japan). The swelling in the foot pad due to the antigens was measured after 24 hours. All procedures took place between 8:00 AM and 12:00 noon.

After seven days, the animals were killed and pancreas and serum were

collected, as described previously. Popliteal lymph nodes were excised, the fat was trimmed, and each lymph node was weighed. Twelve animals from the NIH-fed group and twelve animals from the HC-fed group were used for partial phenotyping of popliteal lymph node cells by flow cytometry. This was kindly performed by Mrs. Heather Cloutier in our laboratory. The popliteal lymph nodes were placed individually in Hank's Balanced Salt Solution. They were then pressed through a 100 mesh stainless steel screen. Cells were counted using an electronic particle counter (Coulter Model ZM, Coulter Electronics, Burlington, Ontario). The cells were aliquoted in three samples at 2.5 X 10^5 cells/ tube in siliconized tubes. The tubes were spun at 150 X g at 5° C and the supernatant was then discarded. The cells were stained with an anti-rat CD5 mouse monoclonal, OX-19, specific to T cells, labeled with fluorescein isothiocyanate (Serotec, Toronto) and an anti-rat leukocyte common antigen mouse monoclonal OX-33 specific to B cells labeled with phycoerythrin (Serotec, Toronto) in PBS containing 5% fetal calf serum in the same tube. The cells were incubated on ice for 30 minutes. They were then washed twice in PBS, spun at 400 X g at 5° C and the pellet was fixed in 2% paraformaldehyde in PBS. The cells were kept overnight at 4° C in the dark and read on a Becton Dickinson FACSCAN. Data were collected per 10,000 events and gated for phycoerythrin or fluoroscein fluorescence.

Experiment 4: Effect of the Exposure of the Neonatal Gut to Diabetogenic Diet or Dietary Components on the Rate and Timing of Diabetes Incidence.

Experiment 4, was designed to investigate the influence of very early exposure of the immature gut to diabetogenic foods. The earliest time at which pups would be exposed to diet, other than those elements of the diet present in breast milk, would be when they begin to feed on their mother's food, around 14 days of age. We were looking at a much earlier time in the neonate's life. A pilot study indicated that early feeding reduced the incidence of diabetes. Two successive experiments were carried out, feeding diabetogenic diets and elements from them to rat pups between the ages of 4 and 7 days. Animals were weaned onto the same diabetogenic diet which was used for the oral dosing. Diabetes incidence, pancreatic inflammation and age at onset were determined. Neonatal BB rat pups were removed from their dams and hand fed daily from 4 days of age to 7 days of age a mixture of Pregestimil® (Mead Johnson, Belleville, Ontario) and ground NIH diet, a mixture of Pregestimil® and wheat gluten, or Pregestimil® alone. The NIH was given in a dose of 60 mg/0.45 ml, the wheat gluten at a dose of 15mg/0.45 ml, to deliver 15 mg protein per feeding. The amount of protein in the NIH slurry fed to the pups was equivalent to 1 mg/g body weight, as was the protein in the other treatments, 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. The diet mixture was kept at body temperature (37°C) 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 four treatment groups. In order to tell them apart at weaning, the pups were tattooed on the foot. After the four days of hand feeding, the pups remained with the dams until weaning at 23 days. The dams were fed a diabetogenic NIH diet. Control, Pregestimil®-treated pups were randomly divided between groups weaned onto the diabetogenic diets, NIH-07 or wheat gluten based diet. Animals treated with NIH were weaned onto NIH-07; those treated with wheat gluten were weaned onto the wheat gluten based diet.

Statistics

Data were analysed using *Statistica* (StatSoft Inc., Tulsa). ELISA results and the data from the popliteal lymph node experiment were analysed using a one-way ANOVA. Insulitis scores were analysed using Student's t test. Diabetes incidence was analysed using Fisher's exact test. Analysis of the survival of neonatally treated animals was carried out using Kaplan-Meier statistics with the log-rank test.

RESULTS

These experiments confirmed the dramatic protective effect of feeding a diabetogen-free hydrolysed casein based diet to BBdp rats compared to rats fed the diabetogenic diets, NIH and WG. (Figure 2). For those animals that developed diabetes, the age of onset was greater on the HC diet. Also the average rating given by microscopic evaluation of the pancreata was significantly lower for those animals on the HC diet. The incidence of diabetes was significantly lower in animals fed the HC diet (Table 1). As can be seen from Figure 3, there were no diet-related differences in the growth rate of the rats. Comparison of the HC diet with each of the diabetogenic diets in terms of diabetes incidence, age at onset or the insulitis rating showed highly significant differences (Table 1; $p<0.0001$).

Experiment 1: The Ability to Establish Oral Tolerance in Antibody and Cell-Mediated Responses to Soluble or Complex Antigens in the BBdp and BBc Rat fed the Diabetogenic NIH Diet or the Protective HC Diet at 35 days of age

Antibody Levels

"Oral tolerance" is used to describe a long recognized phenomenon of antigen specific immunological hyporesponsiveness after prior enteral administration. An animal fed specific antigens will usually show a reduced humoral and cell-mediated immunity to that antigen when challenged subsequently. This decrease in reactivity is dependent on antigen dosage,

Table 1. Effect of Diabetogenic Diet vs. a Protective Diet[†]

Diets	Rats/ group	Diabetes Incidence (%)	p vs. HC	Insulitis Rating	p vs. HC	Average Age at Onset (d)	p vs. HC
HC	8/99	7.8		2.1 ± 0.1		117.1 ±1.5	
NIH	75/144	52.1	p<0.0001	3.0 ± 0.1	p<0.0001	93.4 ± 0.6	p=0.016
WG	31/63	49.2	p<0.0001	2.8 ± 0.1	p=0.0007	87.2± 0.9	p=0.0004

[†]HC diet compared with NIH and WG diets by diabetes incidence (Fisher's exact test), age at onset or insulitis score (Student's t test); values for insulitis and age at onset are $\bar{x}$ ± S.E.

species, strain and age of the animal. Oral tolerance is not always induced: exposure to antigen via the oral route in the neonate can lead to priming of immune responses, not to reduction of the response (Miller, Lider, Abramsky and Weiner, 1994).

None of the antigen treatments affected diabetes incidence or survival of the animals, regardless of the diet fed (Figure 4). Injection of the diabetogenic NIH diet did not accelerate diabetes in NIH fed animals, nor did it increase the incidence of diabetes in animals fed the protective HC diet. BSA, though thought by some to be a trigger of IDDM, did not influence the incidence of diabetes.

The levels of antibodies present after the regime to induce tolerance but

before and then after systemic challenge (44d and 66d respectively) to OVA, BSA, NIH, and SRBC in the sera of the BBdp and the BBc rats fed protective or diabetogenic diets, are summarized in Figure 5.

The groups in which the animals were fed the antigen daily for 5 days showed significant increases in antibody levels to that antigen between the serum samples taken before and after challenge, suggesting a lack of humoral tolerance (Figure 5). However, daily feeding of the NIH diet did produce tolerance in both BBc and BBdp rats, as there was no significant difference in the antibody levels to NIH between the two samplings (Figure 5A). This indicated that in both BBdp and BBc animals, daily exposure to the NIH dietary antigens did induce tolerance. This suggests that the gut immune system is functional in both sublines in limiting the production of circulating antibodies following injection of these dietary antigens. Secondly, results from the animals fed the HC diet demonstrated that there was no problem generating antibodies to elements in NIH. Also a short term exposure to antigens via the gut did not result in a limiting of antibody production following subsequent challenge. BSA is found in skim milk powder, a constituent of NIH, and has been proposed as a diabetes-inducing food antigen that results in abnormally high antibody levels in diabetes prone individuals (Karjalainen et al, 1992). However, BSA did not affect diabetes incidence in either of the sublines. There was a strong antibody response after challenge to BSA. With the exception of NIH antibodies in NIH-

fed rats, antibody response was higher at 66d compared to 44d. At 44d of age, antibody response was highest against complex antigen mixtures (NIH, SRBC) and low against either BSA or OVA. In general, there was no correlation with antibody levels to the antigens used and the survival of the animals.

Histologic Rating of the Pancreata

The qualitative rating of the pancreas of BBdp animals showed a significant difference (Table 1, $p<0.0001$) in the degree of insulitis in the pancreata of animals fed the protective diet, HC (2.1), and the animals fed NIH (3.0). This correlated directly with incidence of diabetes between the two diets and inversely with the age at onset of diabetes. In BBdp rats fed NIH, the insulitis ratings were not significantly different when compared with animals dosed orally with the soluble antigens, OVA and BSA, (Table 2).

Table 2. Pancreas Rating of BBdp Rats fed NIH and Dosed Orally with Various Antigens. (Values are $\bar{x} \pm$ S.E.)

Antigen	n	Pancreas Rating
Saline	11	2.7 ± 0.3
NIH	11	2.7 ± 0.4
OVA	12	3.2 ± 0.3
BSA	12	3.2 ± 0.3
SRBC	12	3.6 ± 0.3

Animals treated with SRBC on NIH diet had the highest insulitis score. The insulitis values parallel the observed incidence of diabetes in the BBdp animals, as discussed above: survival of BBdp rats on HC and on NIH diets was not affected by any of the antigen treatments (Figure 4). Diet was the predominating effect.

Experiment 2: Intrathymic Injection of Dietary Antigens: The Ability to Induce Tolerance to the Islet β Cells by Injection of Diabetogenic NIH Intrathymically, Demonstrated by Inhibition of Diabetogenesis

As the site where T cells are selected, the thymus is central to the production of tolerance to self antigens. Self antigens, presented in the thymus, led to the deletion of self reactive T cells. Injection of foreign islets into the thymus leads to tolerance of islets transplanted from a genetically identical donor (Posselt, Barker, Friedman and Naji, 1992). Injection of islets into the thymus of fetal BB rats leads to life long prevention of diabetes (Posselt, Barker, Friedman and Naji, 1992). So it is possible to manipulate experimentally this central tolerance by exposing the thymus to foreign antigens and so induce tolerance in the animal to these antigens. If something in the diet were activating anti-islet T cells, it might be perceived by the organism as similar to islet antigens, so we hypothesized that early injection of the diet into the thymus might lead to lifelong tolerance to the diabetogenic elements of the diet and so prevent diabetes. This required autoclaving the diet, a treatment that does not

decrease the diabetogenicity of the diet. Thymic injection of autoclaved dietary antigens at 24 hours of age resulted in no change of incidence of diabetes (Figure 6). In the group injected with saline, 12 out of 26 (46.2%) animals developed diabetes. In those injected in the thymus with NIH, 11 out of 27 (40.7%) animals developed diabetes. Insulitis scores were not significantly different between the two groups (Table 3). Thus, neonatal injection of diabetogenic diet into the thymus did not affect the incidence of diabetes or insulitis.

Several animals were injected with India ink and killed to confirm the intrathymic location of the injection (Figure 7). Paraffin embedded 5 μm unstained sections were examined under Nomarski optics. India ink was seen in the thymic medulla, as well as in the thymic cortex, where T-cell selection occurs. Examination of the India ink injected thymi showed that the injections exposed the thymus to the injected material.

Table 3. Rating of the Insulitis in Pancreata of Thymus Injected Rats

Treatment	Insulitis Score ($\bar{x}$ ± S.E.)	Number of Animals Scored
Saline	2.8± 0.2	25
NIH	2.7± 0.2	25

Experiment 3: Popliteal Lymph Node Assay to Assess Immune Reactivity to Food Diabetogenic Antigens in a Th1 Mediated Process.

In this experiment we were examining the ability of the diabetogenic diet, when used as an antigen, to elicit a systemic immune response in BBc and BBdp rats as measured by the popliteal lymph node assay.

In the control animals there was a significant difference in the weight of the left popliteal lymph node draining the footpad injected with NIH between the diets, HC and NIH (Table 4, $p<0.05$). The count of B cells and of T cells as well as the total white cell count of the draining lymph node of the foot injected with the dietary antigens was not significantly different. With the BBdp rat the picture is quite different. Reflecting the lymphopenia in these animals, the BBdp animals showed low numbers of cells in the right popliteal lymph node (injected with the carrier, Pregestimil®) and a decrease of T to B cell ratios. The total white cell count in the left popliteal lymph node was reduced significantly in the NIH fed animals. The percentage of B cells in the lymph node was also reduced. However, the percentage of T cells was increased in the NIH fed rats, 26.9% compared with 22.2% in the HC fed rats (Table 4). None of these effects was seen in the right popliteal lymph node, draining the foot pad which did not receive the NIH mix (Table 4).

The footpad DTH reaction to NIH in the diabetes prone rats showed a significantly smaller increase in swelling in the animals fed NIH compared to

Table 4. Popliteal Lymph Node Analysis using NIH as an Injectable Antigen in BB Rats at 70d [§†]

	BBdp		BBc	
	HC	NIH	HC	NIH
DTH[‡] in Right Footpad (mm)	0.330 ± 0.075[a] (36)	0.155 ± 0.085[a] (35)	0.310 ± 0.080 (36)	0.330 ± 0.075 (35)
DTH[‡] in Left Footpad (mm)	1.716 ± 0.127[b] (36)	1.364 ± 0.131[b] (35)	1.288 ± 0.134 (36)	1.084 ± 0.131 (35)
Sum of DTH in Both Footpads (mm)	2.046 ± 0.140[c] (36)	1.519 ± 0.141[c] (35)	1.598 ± 0.014 (36)	1.414 ± 0.137 (35)
Weight of Right Popliteal Lymph Node (mg)	7.3 ± 3.4 (36)	5.6 ± 2.5 (35)	9.8 ± 3.8 (36)	10.5 ± 4.0 (35)
Weight of Left Popliteal Lymph Node (mg)	15.5 ± 5.3[a] (36)	11.4 ± 4.7[a] (34)	23.8 ± 11.6[b] (36)	17.9 ± 9.5[b] (34)
White Blood Cell Count (X 10^6/ml) in Right Popliteal Lymph Node	1.3 ± 0.4 (9)	1.2 ± 0.5 (10)	7.5 ± 2.9 (13)	6.7 ± 5.6 11
White Blood Cell Count (X 10^6/ml) in Left Popliteal Lymph Node	7.0 ± 0.7[b] (12)	3.9 ± 0.5[b] (12)	23.8 ± 0.8 (13)	20.6 ± 1.1 (11)
T Cell % in Right Popliteal Lymph Node	24.7 ± 5.5 (9)	23.4 ± 5.9 (10)	92.7 ± 2.9 (13)	83.7 ± 7.5 (11)
T Cell % in Left Popliteal Lymph Node	22.2 ± 0.7[b] (12)	26.9 ± 0.7[b] (12)	92.0 ± 0.5 (13)	90.2 ± 0.6 (11)
B Cell % in Right Popliteal Lymph Node	58.8 ± 6.1 (9)	62.2 ± 5.2 (10)	5.6 ± 2.5 (13)	13.1± 6.8 (11)
B Cell % in Left Popliteal Lymph Node	66.6 ± 0.7 [b] (12)	60.5 ± 0.6 [b] (12)	6.6 ± 0.4 (13)	7.8 ± 0.5 (11)
T/B Cell Ratio in Right Popliteal Lymph Node	0.4 ± 0.0.1 (9)	0.4 ± 0.1 (10)	18.9 ± 6.6 (13)	9.0 ± 6.2 (11)
T/B Cell Ratio in Left Popliteal Lymph Node	0.3 ± 0.1[b] (12)	0.5 ± 0.1[b] (12)	16.1 ± 0.7 (13)	14.2 ± 0.9 (11)

[§] All values are means ± S.E. In brackets are the n values for each measure. A Student's t test was carried out within either BBdp or BBc groups of rats. The comparison was only done within the rat type because of the lymphopenia in the BBdp rat. Values with the same superscript are significantly different as described below.

[†] The left footpad was injected with ground autoclaved NIH in Pregestimil®; the right footpad was injected with Pregestimil®.

[‡] DTH measured at 24 hr. The reported values are the change from 0 time. The other measurements were made at seven days.

[a] effect of diet is significant at $p<0.01$.

[b] effect of diet is significant at $p<0.05$.

[c] effect of diet is significant at $p=0.002$

those fed HC ($p<0.05$). However, this effect disappeared when the measure used was the difference between the DTH reaction in left footpad and the right footpad. The weight of the left popliteal lymph node was also less in those animals fed NIH. This effect was also seen in the control animals ($p<0.05$). This effect remained if the measure used was the difference between the right popliteal lymph node weight and the left popliteal lymph node. Also it remained if analysis of variance was carried out using the weight of the right popliteal lymph node as a covariate. This is evidence of an effect of diet on the T cells to induce tolerance in the BBdp rat.

There was a diet effect on the DTH reaction in the right footpad of BBdp rats injected with Pregestimil® only (HC animals: 0.330 ± 0.075 mm; NIH animals: 0.155 ± 0.085 mm; $p=0.02$). The animals fed NIH had a smaller reaction than those fed HC. This indicates a generally suppressive effect of the NIH diet on T-cell mediated reactions. These results showed an effect of the feeding of NIH diet on inducing tolerance in cell mediated immunity, in the DTH reaction and in the measure of changes in the popliteal lymph node, after injection of diet into the footpad. This was seen in the weight of the popliteal lymph node in the control animals, but not in the other measures of cell-mediated immunity; in the popliteal lymph node: the white blood cell count, the T cell to B cell ratio; and in the DTH reaction. In contrast, in the diabetes prone animals, induction of tolerance was seen in the DTH assay, the weight of the left popliteal lymph

node, in the numbers of white blood cells in the left popliteal lymph node, as well as in an increase in the T cell to B cell ratio in the lymph node draining the NIH injected footpad.

Experiment 4: PreWeaning Exposure to Diet: the Effect that the Exposure of the Neonatal Gut to Diabetogenic Diet or Dietary Components has on the Rate and Timing of Diabetes Incidence

Five separate experiments were carried out to examine the effect of pre-weaning oral exposure to antigens associated with diabetogenesis in the BB rat. Three experiments used animals dosed orally from day 4 of life to day 7 with the diabetogenic diet NIH or the vehicle, Pregestimil®. The day of birth, dated as the morning of the recording of the birth, was considered day 1. The animals were then weaned at approximately day 23 onto NIH. A similar design, using a diabetogenic component of the NIH diet, wheat gluten, was used in two other experiments. At 131 d of age, there was a reduction of the incidence of diabetes and a delayed onset in animals pretreated with the diabetogenic diets (Table 5, pooled NIH values). In all three NIH experiments the rate of diabetes appearance and final incidence was less in the BBdp rats dosed neonatally (Figure 8). Because these results were in the same direction and considering the labour intensive nature of these studies which limited the size of individual groups, the data were pooled.

Using Kaplan-Meier estimates to compare the groups, and log-rank

Table 5. Summary of Insulitis Rating, Age of Diabetes Onset and Diabetes Incidence in Individual Experiments and Pooled for Early Oral Exposure to Diabetogenic Food Antigens[†]

Diet/ Trial	Neonatal Dosing	n	Insulitis	Mean Age at Onset (d) ± S.E.	Incidence to age 131 d (%)
NIH 1	Pregestimil®	10	2.9[a]	63 ± 1	60.0[b]
	NIH	8	1.9[a]	98 ± 3	22.2[b]
NIH 2	Pregestimil®	14	2.7	94 ± 2	42.9
	NIH	20	2.3	87 ± 2	23.8
NIH 3	Pregestimil®	15	3.8	79 ± 1	66.7
	NIH	14	3.7	86 ± 2	57.1
WG1	Pregestimil®	12	2.4	80 ± 2	35.7
	WG	19	2.5	97 ± 2	26.3
WG2	Pregestimil®	15	3.5	82 ± 1	73.3
	WG	12	2.8	91 ± 1	40.0
Pooled NIH	Pregestimil®	39	3.1	79 ± 1[c]	56.4
	NIH	42	2.7	88 ± 1[c]	31.0
Pooled WG	Pregestimil®	27	3.0	82 ± 1	51.9
	WG	33	2.7	95 ± 1	33.3
All Treatments Pooled	Pregestimil®	66	3.1[d]	80 ± 1[•]	54.6[f]
	Diet	75	2.7[d]	88 ± 1[•]	32.0[f]

[†] Differences for insulitis and mean age of onset were analysed using Student's t test; differences in incidence were analysed using Fisher's exact test

[a] p=0.04

[b] p=0.04

[c] p=0.02

[d] p=0.05

[•] p=0.003

[f] p=0.01

statistics to evaluate the significance, the pooled results from the NIH studies show a significant effect in delaying the age of onset of diabetes with preweaning treatments NIH (Figure 8, p=0.02). The pretreatment with diabetogenic diet increased the age of diabetes onset. As can be seen from the survival curves of the pooled data (Figure 8), the delay decreased the slope of the curve and resulted in fewer cases of diabetes by the end of the experiment. In two subsequent studies, using wheat gluten as the diabetogen, preweaning exposure to wheat gluten also decreased the rate of onset of diabetes (Figure 9C; p=0.04) in wheat gluten fed BBdp rats. These results, using a single source of diabetogen, itself a component of NIH, confirmed that preweaning exposure to a diabetogen can decrease the rate of onset and reduce the incidence of diabetes. As four out of five of these experiments showed this beneficial pattern, this lends further support to our use of pooled data. In a sixth experiment, this same pattern was also observed with another dietary diabetogen in soy (Rastegar and Scott, unpublished data).

The insulitis score was slightly lower in animals dosed orally with NIH or WG in infancy and this difference was significant when the data from all treatments were pooled (Table 5; Figure 10). This may indicate that the effect of preweaning treatment is in the final stage of the attack on the β cells, possibly changing the quality of the insulitis from Th1 to Th2 cells as reported in other treatments of diabetes (Hancock, Polanski, Zhang et al, 1995) and in HC-fed

BBdp rats (Scott, Cloutier, Kleeman et al, submitted 1996).

The serum IgG antibody levels to NIH or wheat gluten measured in serum collected at necropsy did not indicate any long lasting effect of the exposure to diabetogenic diets at the time of suckling (Table 6); there was no significant difference between animals dosed in infancy with NIH or WG and the control animals treated with Pregestimil® alone.

Table 6. Antibodies to Food Antigens Measured by ELISA Absorbance. Values shown are the pooled results from five experiments in animals exposed to diabetogenic diet components during suckling ($\bar{x}$ ± S.E.). †

Diet	n	Absorbance (405nm)	Treatment
NIH	35	0.230 ± 0.065	Pregestimil®
NIH	32	0.232 ± 0.076	Pregestimil® + NIH
WG	25	0.463 ± 0.120	Pregestimil®
WG	34	0.514 ± 0.104	Pregestimil® + WG

† no significant difference

Summary

These experiments showed that early exposure to diabetogenic antigens in wheat gluten and in the NIH diet (which contains wheat gluten) can delay the onset and reduce the incidence of diabetes in diabetes prone BB rats.

Treatment by daily dosing with antigens for five days at age 35d to 40d

did not induce oral tolerance and had no effect on diabetes outcome. However, continuous feeding of antigens in the diet did induce oral tolerance. The popliteal lymph node experiment demonstrated cell mediated tolerance to NIH in footpad DTH tests and, more robustly, in the weight and constitution of the popliteal lymph node. The change in the weight of the popliteal lymph node seems to be a more sensitive measure of the reaction to an antigen than the DTH reaction itself. The establishment of oral tolerance to diabetogenic dietary antigens in adult animals did not affect either incidence of diabetes or the islet histology. Neonatal exposure to dietary diabetogens did not significantly alter circulating antibodies to the diet constituents. We do not know if oral tolerance was established in these animals, although this treatment delayed and reduced the incidence of diabetes.

Neonatal exposure to dietary diabetogenic antigens in the thymus had no effect on the incidence of diabetes. By contrast, neonatal exposure in the gut to both the diabetogenic dietary antigens in the NIH diet and to the diabetogenic dietary antigens in wheat gluten, a component of NIH, (Table 5; all NIH experiments pooled) reduced the incidence of diabetes in approximately 37% of BBdp rats.

DISCUSSION

It is clear that diet affects the incidence of diabetes in the BBdp rat. In the experiments reported here, a diet whose amino acid source is hydrolysed casein reduced the incidence of diabetes to 7% from 48.5% seen in animals fed an open formula rodent diet, NIH-07. Approximately 85% of our cases therefore can be described as food-induced diabetes. Historically our laboratory has seen rates of diabetes as high as 70% for animals fed the NIH diet (or other mainly plant-based diets). Animals fed the protective HC diet generally have diabetes rates of 15% or less.

The experiments described here suggest that exposure to certain diabetogenic foods in infancy has a protective influence, as much as 37%, on the later development of diabetes in BBdp rats. Although the immune system of the BB rat has been extensively studied, the role of the gut immune system and the effects of various diets in the pathogenesis of diabetes in the BB rat have received little attention.

$RT6^+$ T cells are absent from the systemic immune system. However, they do occur within the mucosal immune system (Fangmann, Schwinzer, Hedrich et al, 1991). The recent discovery that the mucosal addressin appears in the endothelium of the pancreas of the NOD mouse early in life, and again early in the development of diabetes, indicates that there is passage of gut derived lymphocytes through the pancreas (Faveeuw, Gagnerault and Lepault,

1994). Studies of human diabetic pancreas tissue have shown that $CD4^+$ T-cell lines derived from islet-infiltrating lymphocytes in acute-onset diabetes bound to islet endothelium and to mucosal endothelium (Hänninen, Salmi, Simell and Jalkanen, 1993). This demonstrates that islet infiltrating lymphocytes in human IDDM display the phenotype of gut-derived lymphocytes. A possible relationship between diabetes and the gut immune system is suggested by the extensive studies of the modification of diabetes incidence with various diets in the two animal models of spontaneous IDDM, the NOD mouse and the BB rat (Scott, 1994a). The experiments reported here are a first step in understanding that relationship. More particularly, little is known of the effect of infant diet on the immune system in diabetes. Our results demonstrate that more attention should be paid to this area.

The experiments reported here on the influence of dietary antigens on the immune system of the BB rat show that the site of exposure to these antigens is important. Direct inoculation of NIH antigens into the thymus of the neonate had no effect on the development of diabetes. Subcutaneous injection of the diabetogenic diet did not protect against diabetes, nor did it accelerate the disease. Importantly, the injection of diabetogenic diet did not abrogate the protective effect of the hydrolysed casein diet. Only long term exposure from weaning onward to dietary diabetogens in the gut increased the incidence of diabetes. Only oral exposure in the neonatal period to the diabetogenic diet via

the gut protected against diabetes. Avoidance of the dietary exposure to the diabetogenic diet by feeding the protective HC diet reduced the incidence of diabetes more than neonatal exposure to the diabetogenic diet.

The normal physiology of the gut allows some passage of macromolecules. Lymphoid structures in the wall of the intestine, the Peyer's patches, are overlaid with specialized enterocytes, the M-cells. These have reduced lysosomal enzyme activity, smaller microvilli, reduced glycocalyx and mucus, and specialized endocytic mechanisms which allow sampling of the contents of the lumen. Macromolecules are passed across the M cells and transported into the extracellular area around the lymphoid tissue within the Peyer's patch. The macromolecules are processed by antigen presenting cells: dendritic cells, macrophages, and B cells, leading to the activation of the mucosal immune system. Either at these sites or through the normal enterocyte (Cornell, Walker and Isselbacher, 1971), a small fraction of the macromolecules gain access to the systemic circulation. Some molecules are selectively taken up whole. In the mammalian neonate there are Fc receptors in the intestine which aid in transport of milk derived immunoglobulins (mostly of the IgA type) into the circulation. In the rat these are downregulated at weaning, around 23 days of age. Enterocytes express MHC class II molecules in their basolateral membranes, as well as near but not in their apical membranes (Sanderson and Walker, 1995). They have the capacity to present antigens to lymphocytes within and beneath

the epithelium. A known effect of the feeding of antigens is the suppression of the immune response to those antigens.

This "oral tolerance", or more appropriately "mucosal tolerance" (as similar effects occur at other mucosal surfaces such as nasal and bronchial passages) has been proposed as a method of treating autoimmune diseases. By feeding a subject an antigen which is known to be an autoantigen in the disease, such as myelin basic protein in the case of experimental allergic encephalitis, the disease can be reduced in severity (Miller, Lider, Al-Sabbagh and Weiner, 1992). This has been demonstrated in the NOD mouse model of diabetes by feeding insulin (Zhang, Davidson, Eisenbarth and Weiner, 1991). On the other hand, the failure of oral tolerance can be part of the pathogenic process, as can be seen in the case of NZB X NZW F_1 mice (Carr, Tilley, Forsyth et al, 1987) and in the case of IgA nephropathy (Gesualdo, Lamm and Emancipator, 1990). The mechanisms involved in this process are not completely known. Humoral tolerance can be different in its duration or its establishment from cell mediated tolerance (Strobel and Ferguson, 1987). The age of the animal affects the establishment of oral tolerance, with tolerance more successfully induced in younger animals (Faria, Garcia, Rios et al, 1993). Antigen dose or frequency of dosing can also affect the establishment of tolerance. The ability to establish tolerance is dependent on the species and strain of animal.

In the case of the BB rat, we have seen that oral tolerance could not be established in either the BBdp or the BBc subline when using daily exposure to the selected antigens for only five days in 35d old animals. However, if an antigen mixture such as NIH was continuously fed to either of these animals, humoral tolerance to these antigens was established. In experiment 3, the popliteal lymph node test did indicate a smaller increase in size of the lymph node in animals fed the antigen. In that experiment, the DTH reaction is meaningfully depressed in the footpad injected with NIH only if the footpad injected with the carrier is not used as a covariate. This indicates that the distribution of variation in the footpad swelling in the foot injected with dietary antigen and the foot injected with carrier is the same. Indeed, the sum of the swelling in the right footpad, injected with carrier, and the left footpad, injected with NIH, is highly significantly reduced in animals fed NIH ($p=0.002$) compared to animals fed HC. This is not observed in the BBc animals treated in a similar fashion. NIH diet seems to have a generally suppressive effect on the DTH reaction. However, since the popliteal lymph node assay showed that there was significantly less increase in size of the lymph node draining the foot injected with the diabetogenic diet, including when controlled for by the size of the lymph node draining the carrier injected foot, we can conclude that tolerance was induced in the T cell compartment.

Oral tolerance in the BBdp and BBc rats is thus seen to be difficult to

establish. It requires continuous feeding of large doses of the antigen. With the humoral reaction to the antigens other than NIH, tolerance was not established. As the antigen in this case is the diabetogenic diet, NIH, and diabetes is thought to involve a similar Th1-mediated process as the DTH reaction, it is particularly relevant that cell mediated immunity can be established to the diabetogenic diet.

Neonatal thymic injection of the diabetogenic NIH diet did not protect against diabetes. At that age the thymic defect likely responsible for the lymphopenia of the BB rat is not yet evident (Doukas, Mordes, Swymer et al, 1994). It is possible that the stimulation of pre-existing β-cell autoreactive T cells occurs later in life, closer to the time of the onset of the disease. The way in which a DTH reaction to insulinoma cell membranes parallels the course of diabetes in BB rats suggests that this is the case (Voorby, Jeuken and Drexhage, 1990). As the prodromal period of IDDM is extensive (in the BB rat, the first sign of disease, the hyperexpression of MHC class I molecules, occurs at 25 days but diabetes only appears at ≥ 60 days), and as neonatal treatment with islet cells prevents the disease, indicating that tolerance due to deletion of β cell reactive T cells can be established (Posselt, Barker, Friedman and Naji, 1992) it is likely that the failure to induce tolerance by intrathymic injection of NIH is not due to the absence of autoreactive thymocytes. The NIH antigens may not persist long enough in the thymus. Or it may be because the antigen processing in the gut is different from that in the thymus. It is unlikely that NIH

antigens would reach all areas of the thymus. There would then be a chance for some thymocytes to escape negative selection. As intrathymic injection has been used to screen for islet autoantigens important in diabetogenesis (Gerling, Atkinson and Leiter, 1994), our data indicate that the diet diabetogens are not mimics of islet antigens, at least in the context in which they are presented in the thymus.

The experiment on the popliteal lymph node assay cannot give us information on the rate of diabetes in animals with established tolerance in cell mediated immunity because the animals were killed to perform the assay. It is likely that animals fed the diabetogenic NIH diet have established tolerance to it in both the humoral arm and the cell mediated arm of the immune system. This did not affect the incidence of diabetes. Nor does the presumed tolerance of the animals to NIH in past experiments. In spite of an induction of tolerance to NIH presented to the immune system orally, that same route of presentation results in a high level of diabetes. The first experiment clearly showed a reduction in antibody response to NIH, compared to animals fed HC. The humoral response is likely reduced because of oral tolerance.

The DTH reaction is driven by the same Th1 cytokine pattern as the attack on the islet β cells. In BBdp rats fed NIH and injected with SRBC there was less of a reaction than in BBdp rats fed HC. BBdp rats fed NIH had a smaller reaction in the footpad injected with Pregestimil® than those fed HC. The

NIH diet suppressed this Th1 cell-mediated reaction. It has been shown that the reaction to islet cell components in a DTH reaction parallels the progress of diabetes in BB rats (Voorby, Jeuken and Drexhage, 1990). In the experiment using the popliteal lymph nodes, the DTH reaction to NIH in animals fed HC diet was much stronger in BBdp rats than in BBc rats. The popliteal lymph node was not as large in the BBdp animals. In spite of the less competent immune system, the BBdp animal displayed a stronger reaction to NIH than did the BBc animals when it was used as a systemic antigen to which the animal was immunologically ignorant. In animals which had been fed NIH, the popliteal lymph node cells were not as activated. So this Th1 reaction to NIH antigens could be diminished without affecting the Th1 driven diabetogenesis. Unlike the DTH reaction to islet cells (Voorby, Jeuken and Drexhage, 1990), the footpad DTH reaction to diabetogenic diet antigens does not parallel diabetogenesis. This would argue against the food diabetogens being islet cell antigen mimics which can be recognized as such at a site in the skin, as is the case with insulinoma cell membranes. Therefore interaction of the gut with the diabetogenic diet is crucial. This does not preclude the possibility that the non-diabetogenic diet helps to ameliorate the immune dysfunction of the BB rat.

The interest in cow milk as a risk factor for IDDM has led to a number of studies of the level of antibodies to cow milk proteins in diabetic children (Gerstein and VanderMeulen, 1996). While these studies do not consistently

find elevated levels of antibodies to BSA, as was expected, there were elevated levels of antibodies to other proteins. Specifically, antibodies to cow β-lactoglobulin were consistently and significantly elevated in these studies (Savilahti, Saukkonen, Virtala et al, 1993; Dahlquist, Savilhti and Landin-Olsson, 1992; Savilahti, Åkerblom, Tainio and Koskimies, 1988). Cellular immune response to cow β-lactoglobulin was also elevated (Vaarala, Klemetti, Savilahti et al, 1996). These authors point out that IDDM may be associated with a defect in the development of oral tolerance as was previously suggested (Scott, 1994a). In both the BBdp and the BBc rat we have found that oral tolerance requires continuous exposure to dietary antigens to be induced. Cell mediated tolerance is more difficult to induce and more easily abrogated. Difficulties in the induction of oral tolerance may be another point of similarity of the BB rat model to human IDDM.

Early feeding of antigen in animal models has been found to cause priming of the humoral response and not tolerance. Feeding myelin basic protein is protective against experimental allergic encephalomyelitis in the adult. However, in the neonate, it leads to an accelerated disease (Miller, Lider, Abramsky and Weiner, 1994). The protective effect found in the BB rat may shed some light on the current debate on IDDM risk and early feeding practices in the human infant (Gerstein, 1994; Karjalainen et al,1992). Our findings of a protective effect due to diet exposure run counter to the hypothesis that dietary

exposure to putative islet cell cross-reactive antigens early in life increases the risk of IDDM in humans. When autoclaved diet was injected into the neonatal BBdp rat thymus, there was no protective effect from subsequent diabetes development when compared to saline injected animals. Injection of islet cells into the thymus in similar experiments (Posselt et al,1990; Posselt et al,1992) does lead to protection from diabetes. Transplanted islet cells require the presence of donor antigen presenting cells to induce tolerance (Oluwole, Jin, Chowdhury and Ohajekwe, 1994). However, soluble antigens can be presented in the thymus (Oluwole, Chowdhury, Jin and Hardy, 1993). The processing of antigens in the gut lumen and epithelium may lead to different peptides being presented than in the thymus. It is known that serum proteases can break down proteins into peptides and amino acids which are presented in a different MHC context than if the whole protein were taken up into the presenting cell (Falo, Colarusso, Benacerraf and Rock, 1992).

It has been shown that early exposure to soybean proteins in the pig (at three weeks of age) leads to a high titre of antibodies to soybean proteins, similar in magnitude to a systemic injection (Bailey, Miller, Telemo et al, 1993). Yet challenging these pigs with soybean antigen led to no secondary humoral response. The authors felt that this priming of the humoral response led to later tolerance to the antigens in the soymeal. We do not know if our animals showed a similar priming. However, these rats were exposed to the diet much earlier

than the pig. As this is certainly earlier than gut closure, the effects noted may be due to the effect of dietary antigens on the immature gut. The postnatal gut is more permeable to macromolecules than the mature gut, although it is clear that a small proportion of macromolecules will always pass undigested into the systemic circulation (Sanderson and Walker,1995). Proteins are digested through intracellular processes in the immature gut and not mainly in the lumen as in the mature gut (Henning, Rubin and Shulman, 1994). It is possible that some of these proteins can be presented via the MHC class I route and so alter the T cell repertoire (Kovacsovics-Bankowski and Rock,1995). The exposure of the neonatal rat gut to dietary antigens which promote the expression of IDDM in the juvenile and adult BB rat inhibits that development. This is not linked to the establishment of oral tolerance to the dietary antigens, as we could see no difference in the DTH nor in the popliteal lymph node assay nor in the levels of antibody to the diabetogenic diet between animals exposed early in life to the diabetogenic diet. Nor is it comparable to the exposure of similar antigens in the thymus of neonatal rats.

Neonatal exposure to myelin basic protein, the causative antigen in an animal model of multiple sclerosis, leads to priming to the antigen and worse disease later in life (Miller et al, 1994), unlike the protective effect of adult feeding of myelin basic protein (Miller, Lider, Al-Sabbagh and Weiner, 1992). This is the reverse of the pattern which we report here where early exposure

protects against later disease. The repertoire of the gut associated T cells may be permanently altered by the early exposure to antigen. As the expansion of cells from the mesenteric lymph nodes appears to be the source of diabetogenic cells in the thymectomized irradiated model of diabetes (Fowell and Mason,1993), neonatal oral exposure may limit the increase in pathogenic T cells. The diabetes protective casein-based diet was found to increase the T cell count in the draining lymph nodes of the gut, the mesenteric lymph nodes (Field, 1995) in diabetes prone BB rats.

It is intriguing that recent studies of the mouse implicate the expression of the mucosal addressin in the endothelia of the pancreatic islets as an early event in diabetogenesis (Faveeuw, Gagnerault and Lepault, 1994). Early feeding induces the expression of MHC class II molecules in the mouse (Sanderson, Ouelette, Carter and Harmatz, 1993). The rat normally develops MHC class II expression on the intestinal epithelium in the fourth week of life; that is, in the week following weaning (Mayrhofer, Pugh and Barclay, 1983). The introduction of diet probably has a trophic effect on the young intestinal epithelium. The induction of MHC class II molecules on the neonatal enterocytes may allow presentation of antigen to gut immune cells. This precocious development of the neonatal gut under the influence of the early introduction of a complex diet probably has a role in the delay in diabetes we see in our study.

In the NOD mouse model, induction of tolerance to an islet specific antigen in the neonate leads to a delay of onset and reduction in the incidence of diabetes (Peterson, Pike, McDuffie and Haskins, 1994). In the BB rat, early treatment with a lipid autoantigen, sulfatide, leads to a delay in diabetes occurrence but not in incidence (Buschard, Hageman, Hansen and Fredman, 1995). These effects are similar to our results. They were obtained by inducing tolerance by direct injection of the autoantigen. This method of neonatal tolerance is known to enhance Th2 cytokines and diminish Th1 cytokines (Chen and Field, 1995). Th1 cytokines are associated with the diabetes lesion (Rabinovitch, Suarez-Pinzon, El-Sheikh et al, 1996; Scott, Cloutier, Kleemann et al, 1996; Zipris, Greiner, Malkani et al, 1996). Soluble proteins in the circulation tend to produce tolerance, not priming. Our results might be explained then as a form of tolerance to antigens which pass directly into the circulation of the neonatal rat. This would then indicate that the dietary antigens act in a fashion similar to the autoantigens in the mouse and rat model discussed above. We have not yet attempted to recover dietary antigens from the serum of neonates fed NIH or WG during suckling.

Alternatively, early exposure of antigen may cause the neonatal rat to precociously develop a competent gut immune system which then is responsible for the delay in diabetes onset. If the result of early feeding is not diabetogen specific, feeding one diabetogenic diet to the neonate might protect against

another diabetogenic diet fed after weaning. If the result of early feeding is non-specific, feeding a complex but non-diabetogenic diet, such as fishmeal, to the neonate would be protective for an animal weaned onto a diabetogenic diet.

It is possible that the administration of a complex food to the neonatal rat has metabolic effects at a time when the β cell is immature. Insulin production is unresponsive to glucose in the neonate, although it is produced in response to arginine. Exposure to glucose sensitizes the β cell, stimulating the insulin secretion response. In a study on the neonatal BB rat, stimulation of the β cell with intraperitoneal glucagon with glucose, or arginine with glucose, led to a reduced incidence of diabetes and later onset (Buschard, Jorgensen, Aaen et al, 1990). These authors point out that responsiveness to glucose is a characteristic of newborn infants of diabetic mothers. In the NOD mouse, neonatal intraperitoneal injections of glucose with arginine led to increased expression of islet autoantigens in the neonatal pancreas. This treatment led, later in life, to an increase in diabetes incidence (Senecat, Martignat, Elmansour et al, 1994). It was felt that neonatal stimulation of the β cell led to earlier exposure of autoantigens associated with insulin secretion and this led to an acceleration of the disease. However the same phenomenon in the BB rat leads to protection from diabetes, likely through early peripheral encounters by T cells with β cell antigens. Differences like these between the BB rat and the NOD mouse are known to occur (Senecat, Martignat, Elmansour et al, 1994).

Children of type I diabetic mothers have a lower incidence of diabetes than do children of diabetic fathers (Warram, Krolewski, Gottlieb and Kahn, 1984). It is likely that the mother's hyperglycemia induces a similar precocious development of the fetal β cell. Neonatal β cell stimulation is likely to increase the expression of islet cell autoantigens. Why this would have the effect of increasing the risk of diabetes in the NOD mouse yet be protective in the BB rat and in the human is unclear. Intrathymic transplantation of neonatal islets does not induce tolerance whereas similar transplantation of adult islets does induce tolerance (Ketchum, Moyer, Pan et al, 1995). This is attributable to the lack of immunogenicity of the neonatal islets, and possibly to the lack of passenger MHC II antigen presenting cells. It is possible that what we have seen in our animals is a result of indirect neonatal β cell stimulation. If this is the case, it is unlikely that passage of glucose or free arginine into the blood stream would explain these effects. These are likely to be quickly cleared by the liver. There is unlikely to be a large difference in the amount of these nutrients contributed by NIH or wheat gluten in Pregestimil® when compared to Pregestimil® by itself. It is more likely that a β cell stimulus comes from hormones secreted by the intestine in response to feeding.

One likely candidate is glucagon-like peptide (GLP-1) (Creutzfeldt and Nauck, 1992). This is released by the intestinal L-cells after feeding. It is a potent insulin secretagogue and it inhibits glucagon release (Dalessio, Prigeon

and Ensinck, 1995). Recently it has been demonstrated to be an important signal of satiety to the brain (Turton, Oshea, Gunn et al, 1996). More importantly, in a study of human fetal β cells, GLP-1 induced glucose stimulated insulin secretion, just as glucagon did in the rat experiment (Otonkoski and Hayek, 1995). There does not appear to be any reason for a differential release of GLP-1 in animals fed NIH or wheat gluten in Pregestimil® when compared to Pregestimil® by itself, although it is possible that these more complex nutrients may stimulate the precocious maturation of the gut. When unusually large amounts of nutrients are delivered to the distal small intestine, an excess of the enteroglucagons, including GLP-1, is released (Walsh, 1994). Gastric inhibitory polypeptide is also an insulin secretagogue released by the gut, although it is not as potent as GLP-1. It may play a role in this process. So our hand feeding of the complex foods might have resulted in such a situation, whereas the Pregestimil likely was quickly digested. At this point, neonatal stimulation of β cells is a possible explanation of our results.

Another possibility is that early introduction of diet allows more of the antigens from the diet into the systemic circulation, to which the animal becomes tolerized similarly to neonatal tolerance. Gut closure occurs over the period from 14d to 21d in the rat and introduction of dietary antigens before 14d delays the development of gut closure (Arvola, Rantala, Marttinen and Isolauri, 1992). This is not due to a loss of integrity of the gut. Absorbed macromolecules are

transported transcellularly. Early introduction of complex dietary antigens increases the amount of intact protein which survives the transcellular route without being degraded. At the time of gut closure the rat immune system is still immature. So the introduction of dietary antigens before gut closure likely accelerates the maturation of the immunological competence of the mucosal immune system while exposing the immature systemic immune system to more intact dietary antigens. It is unclear how this protects against diabetes. It may be a specific effect of dietary antigens mimicking islet autoantigens or it may be a more general effect such as hormonal as discussed above. Certainly, the induction of oral tolerance to the diabetogenic diet and the lack of effect on the incidence of diabetes in adult animals demonstrates that the protection is time as well as site specific.

It is intriguing that the early exposure to a diabetogenic diet like NIH can delay the onset of diabetes. NIH will trigger the development of diabetes in the BB rat even if exposure is delayed until the animal is 50 days of age (Scott,1994; Scott et al, submitted, 1996). In the few animals per group which were tested, we saw no evidence of priming of the antibody response to dietary antigens in animals killed at weaning. The effect of preweaning exposure to diabetogenic dietary antigens is probably mediated through the T cells. The effect is long lasting as later development of diabetes is either delayed or prevented by this treatment. Exposure to a diabetogenic diet after weaning and as late as puberty

potentiates the development of diabetes. Neonatal exposure dampens down the process, even preventing it entirely in some animals.

At the time of exposure to the diet in these studies, at day 4 to day 7, the number of $CD8^+$ T cells in the lymphoid organs of the BB rat is not different from those in the Wistar rat (van Rees, Voorbij and Dijkstra, 1988). The early exposure to dietary diabetogens may alter the development of the T cells. It may be as in oral tolerance to insulin in the NOD mouse, that cells secreting Th2 cytokines migrate to the pancreas (Hancock et al., 1995). Whatever role early introduction to diet plays, it is salient that at this age of exposure neither the thymic defects nor the general deficiency in T cells characteristic of the BB rat are evident. Diet may help preserve cells otherwise lost in BB rat development. More work needs to be done on the mechanism of prevention of diabetes by neonatal exposure to diabetogenic diets.

In summary, early introduction of diabetogenic dietary antigens to the BB rat delays onset and reduces incidence of diabetes. Evidence of tolerance to the diabetogenic dietary antigens can be found in animals fed the diabetogenic diet but this is not associated with a decrease in diabetes but rather an increase. Animals fed a protective HC diet and injected in the footpad with diabetogenic NIH dietary antigens showed higher T cell and B cell responses to the NIH but showed no increase in diabetes. A recent report on the feeding of Pregestimil® to NOD mice found, that while this hydrolysed casein based formula protected

against diabetes, there were no obvious diet-related immunologic changes observed (Hermitte, Atlangepner, Payan et al, 1995). Oral tolerance, as seen in the suppression of the antibody response after challenge to fed antigens, and in the reduction of popliteal lymph node increase in animals fed NIH, which we infer implies the establishment of cell mediated tolerance in the animals fed NIH in our other experiments, does not protect against diabetes, but neonatal feeding of a diabetogenic diet, perhaps associated with priming (Weiner, Friedman, Miller et al, 1994), has a delaying and partially protective effect. As we see it, there are three possible mechanisms to explain the delay in onset and the reduction in incidence in diabetes in diabetes prone BB rats exposed early in life to a diabetogenic diet: 1) hormones secreted by the gut cause the earlier exposure of the immune system to islet cell autoantigens, leading to tolerance in some undefined way, but similar to the effect in induction of tolerance by early exposure to the islet autoantigens, sulfatide (Buschard, Hageman, Hansen and Fredman, 1995) and glutamic acid decarboxylase (Petersen, Karlsen, Markholst et al, 1994); 2) precocious maturation of the gut immune system in response to diabetogenic antigens, causing lymphocytes derived from the gut to home to the pancreas, inducing tolerance in the lymphocytes to the β cells; or; 3) the early leakage of diabetogens contained in the diet from the gut into the circulation leading to tolerance induction akin to the intravenous administration of soluble protein.

Insulitis occurs in some HC-fed animals which do not develop diabetes. A shift in cytokine profiles within the islet from Th1 predominating to Th2 has been seen in this situation (Scott, Cloutier, Kleemann et al, 1996). It is also possible that a greater ability to regenerate destroyed β-cell mass may account for the differences in survival among our groups. In the transgenic mouse model with interferon-γ secreting β-cells, a longer prediabetic stage in the female mouse compared to the male mouse was ascribed to the greater regenerative capacity of the former (Gu, Molony, Krahl and Sarvetnick, 1995). Tolerance induction may be controlled in the periphery by the amount of antigen present. It has been found that insulitis, the distinguishing lesion of autoimmune diabetes, can be induced in the normal rat by massive pancreatectomy (Lampeter, Tubes, Klemens et al, 1995). Autoimmune attack could be prevented by subcutaneous injection of a rat islet homogenate. So the actual amount of islet cell antigen present controls the level of tolerance.

There are indications that HC diet exerts its protective effect at least in part by increasing islet mass (Scott, Cloutier, Kleemann et al, 1996). Massive pancreatectomy encourages islet cell regrowth. The islets in these animals are much larger than normal, indicating (Lampeter et al, 1995) that islet inflammation itself encourages regeneration of islets. We have often seen enlarged islets in the surviving animals but it does not appear to be a consistent feature. However, an early induction of islet inflammation in the preweaning diabetogenic

diet treated animals might result in a larger islet mass later in life, when peripheral tolerance is broken in diabetogenesis. This increased level of antigens probably increases the chance for tolerance to the islet antigens to be maintained (Ferber, Shonrich, Schenkel et al, 1994).

These hypotheses could be distinguished by: 1) investigating the effect of GLP-1 administration to the neonatal BB rat; 2) blocking the mucosal addressin by administering an antibody to it at the same time as neonatal exposure to the diabetogenic diet, to see if the protective effect is abrogated; and; 3) direct intravenous inoculation of soluble extracts of the diabetogenic diet, such as the elements of the wheat gluten portion, in the neonatal rat, to see if diabetes could be delayed.

These results indicate that immunological effects of environmental influences such as diet must be considered in the etiology of diabetes. Protective diets may be feasible for humans at risk. We find that in an animal model of diabetes the feeding of diabetogenic diets to neonates reduces the incidence and increases the age of onset of diabetes. These findings should focus attention on the role of mucosal tissues in autoimmune disease and the relevance of the early exposure to antigens.

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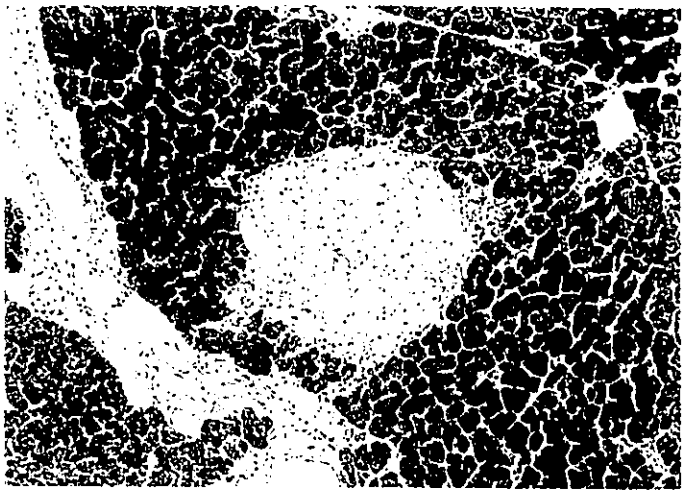
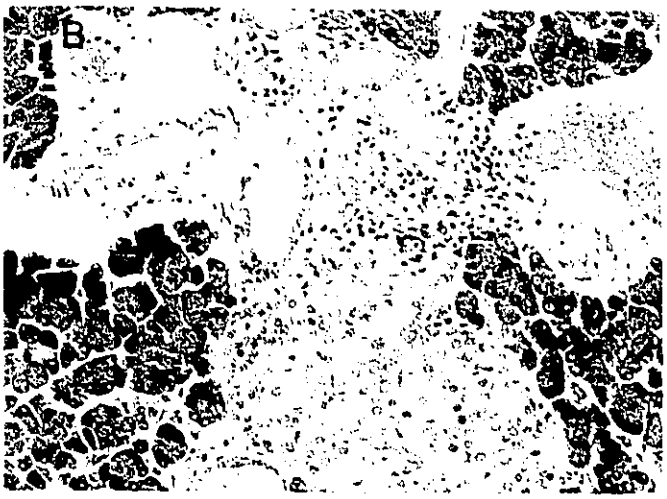
FIGURES

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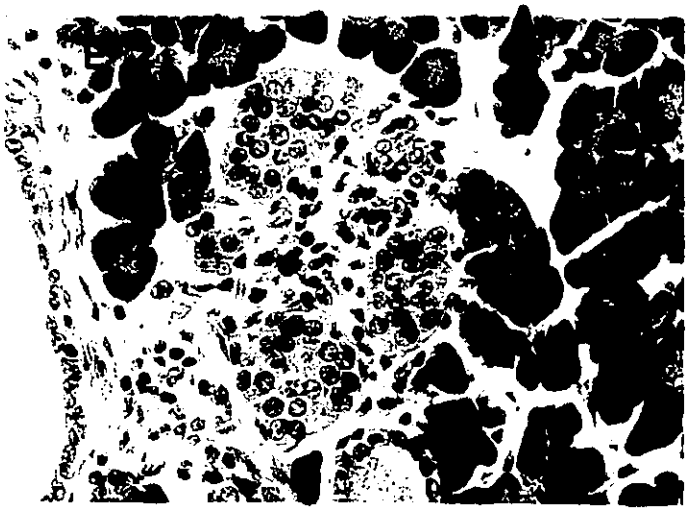


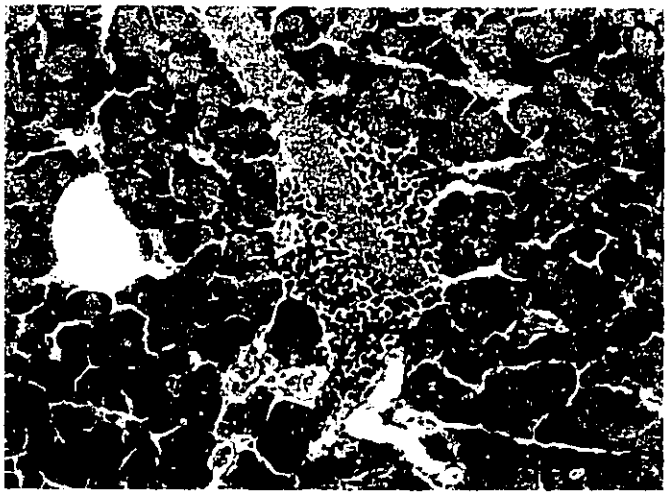


Figure 1. Rating of the islet lesions in BBdp rats
A. Islet from a pancreas rated 1. 109 X. Hematoxylin and eosin stain (H&E).
B. Islet from a pancreas rated 2. 109 X. H&E.
C. Islet from a pancreas rated 3. 109 X. H&E.
D. Islets from a pancreas rated 4. 218 X. H&E.
E. Islet from a pancreas rated 5. Note the small size and predominance of α cells. 435 X. H&E.
F. Perivascular infiltrate. 218X. H&E.

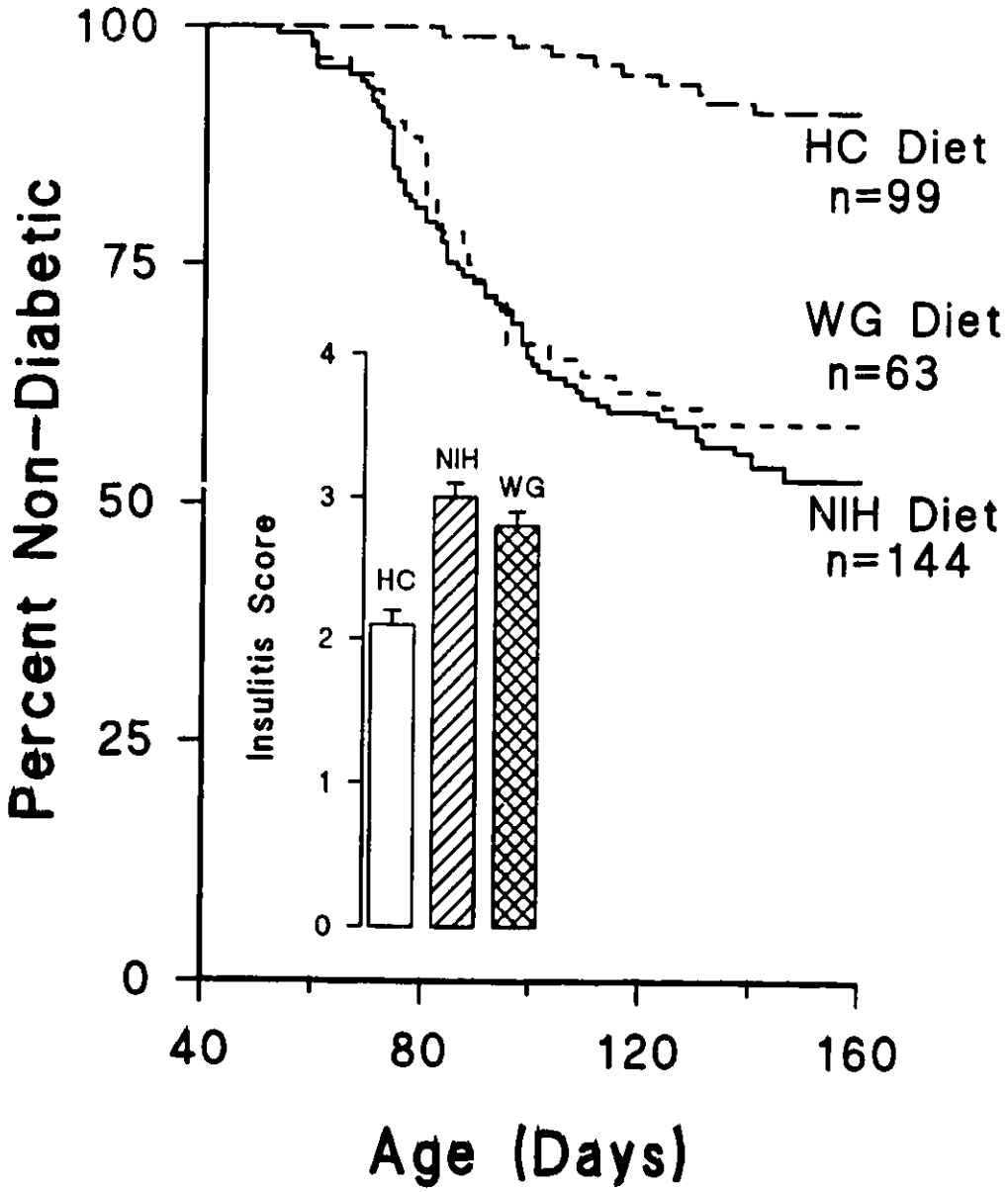

100
75
50
25
0
Percent Non-Diabetic
40
80
120
160
Age (Days)
HC Diet
n=99
WG Diet
n=63
NIH Diet
n=144
Insulitis Score
4
3
2
1
0
HC
NIH
WG

Figure 2. Dietary control of autoimmune diabetes in the BBdp rat.
Summary of the percentage of animals surviving on a protective hydrolysed casein based diet (HC) and on diabetogenic diets: an open formula rodent diet based mainly on plant materials (NIH), and a diet based on wheat gluten (WG). Summary of seven experiments.
Inset shows the qualitative rating of the histology of the pancreas from each treatment, based on a score of 1-5 ($\bar{x} \pm$ S.E.). Normal appearing islets were rated as 1; 2 indicated mild, 3 a majority of islets infiltrated, 4 most islets heavily infiltrated and 5 indicated endstage disease, with few islets, and those appearing to contain only alpha cells, with little inflammatory infiltrate remaining.

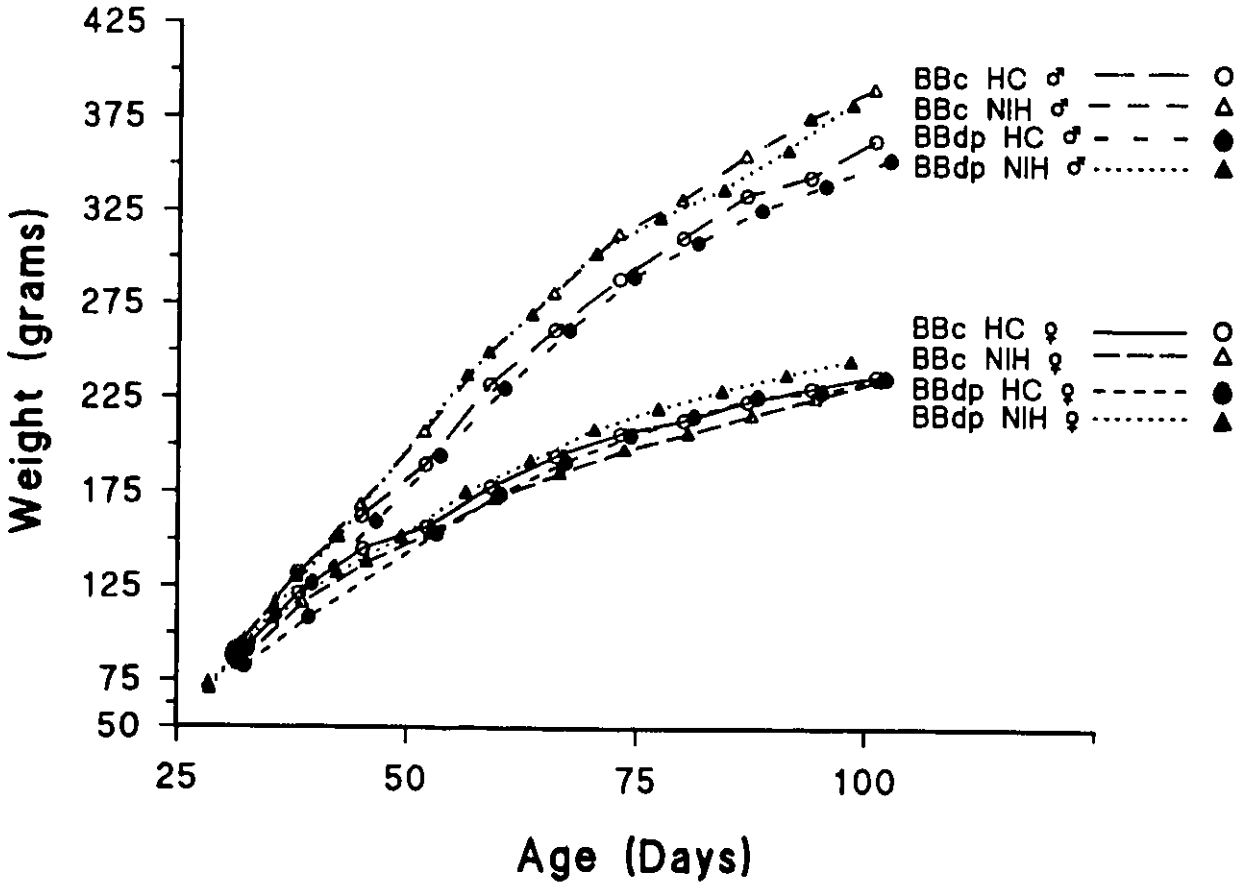

425
375
325
275
225
175
125
75
50
Weight (grams)
25
50
75
100
Age (Days)
BBc HC ♂
BBc NIH ♂
BBdp HC ♂
BBdp NIH ♂
BBc HC ♀
BBc NIH ♀
BBdp HC ♀
BBdp NIH ♀

Figure 3. Growth rate of diabetes-prone BBdp and diabetes-resistant BBc rats on the protective HC diet and the diabetogenic NIH diet.
Weights and ages for each data point are averaged across all animals of each gender and type for each diet. (n≈30).

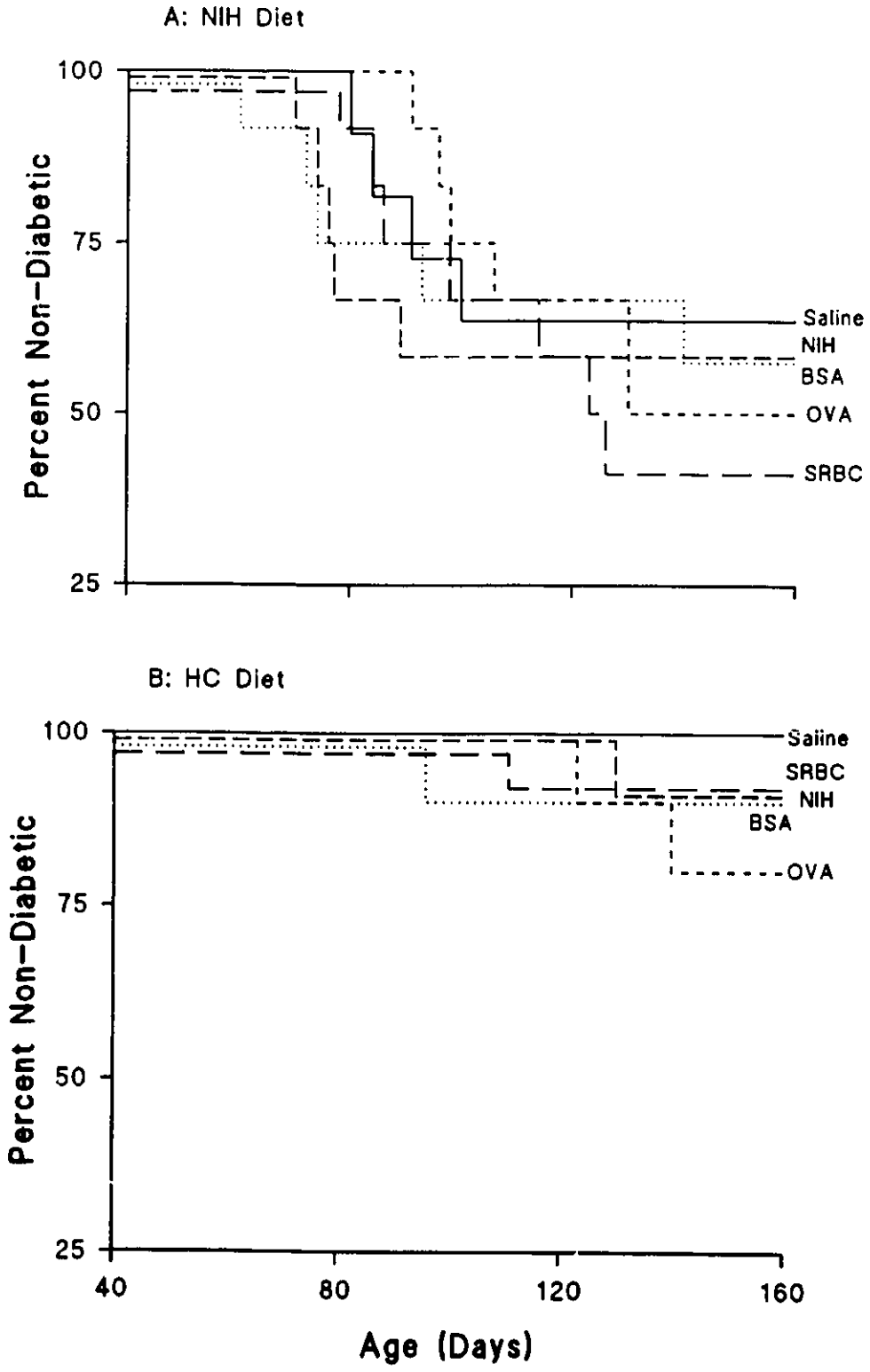
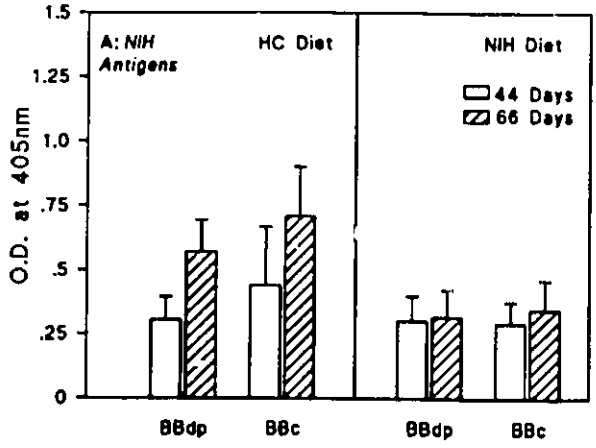
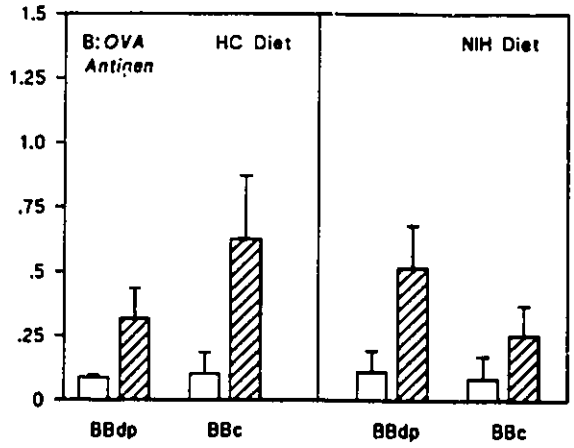
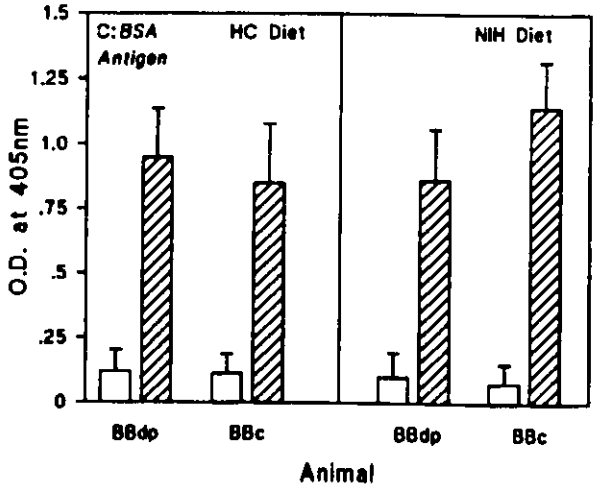

A: NIH Diet
100
75
50
25
Percent Non-Diabetic
Saline
NIH
BSA
OVA
SRBC
B: HC Diet
100
75
50
25
Percent Non-Diabetic
Saline
SRBC
NIH
BSA
OVA
40
80
120
160
Age (Days)

Figure 4. Effect of antigen treatment on the survival of BBdp rats.
Animals were fed the antigen daily by gavage between days 35 and 40 of age. They were challenged with antigen in complete Freund's adjuvant at 45 days of age and again in incomplete Freund's adjuvant for DTH reaction at 66 days.
A. Animals fed the diabetogenic NIH diet.
B. Animals fed the protective HC diet.
n=12 BBdp rats/group.


A: NIH Antigens
HC Diet
NIH Diet
44 Days
66 Days
O.D. at 405nm
1.5
1.25
1.0
.75
.5
.25
0
BBdp
BBc
BBdp
BBc


B: OVA Antigen
HC Diet
NIH Diet
1.5
1.25
1.0
.75
.5
.25
0
BBdp
BBc
BBdp
BBc


C: BSA Antigen
HC Diet
NIH Diet
O.D. at 405nm
1.5
1.25
1.0
.75
.5
.25
0
BBdp
BBc
BBdp
BBc
Animal

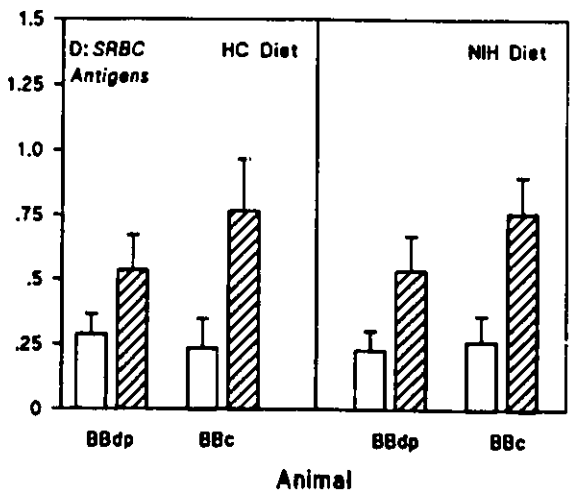

D: SRBC Antigens
HC Diet
NIH Diet
1.5
1.25
1.0
.75
.5
.25
0
BBdp
BBc
BBdp
BBc
Animal

Figure 5. Serum antibodies to antigens before and after challenge.
Relative antibody production to NIH antigens in animals fed a hydrolysed casein based diet (HC) and fed an open formula rodent diet based on plant materials (NIH). IgG serum antibody levels measured by ELISA using ABTS on serum samples collected by orbital bleeds. Animals were fed antigen daily by gavage between days 35 and 40 of age. They were challenged with antigen in complete Freund's adjuvant at 45 days of age.
n=12 BBdp or BBc rats/group.

A. Diabetogenic NIH-07 diet (NIH) used as an antigen.
B. Ovalbumin (OVA) used as an antigen.
C. Bovine serum albumin (BSA) used as an antigen.
D. Sheep red blood cells (SRBC) used as an antigen.
($\bar{x} \pm$ S.E.)

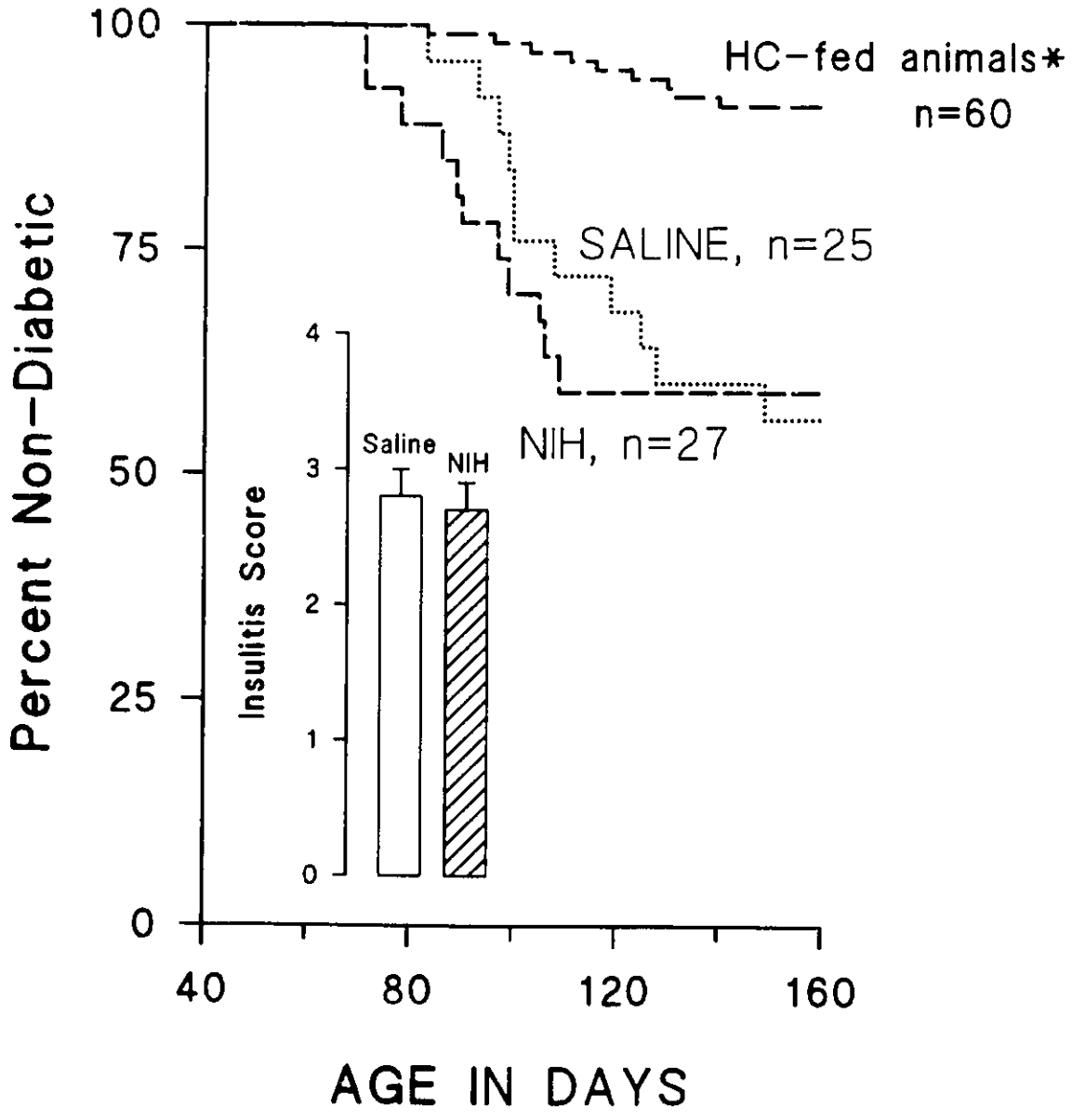

100
75
50
25
0
Percent Non-Diabetic
HC-fed animals*
n=60
SALINE, n=25
NIH, n=27
Saline
NIH
Insulitis Score
4
3
2
1
0
40
80
120
160
AGE IN DAYS

Figure 6. Survival of BBdp rats injected with NIH in the Thymus.
Effect of intrathymic injection of autoclaved diabetogenic NIH-07 diet (NIH) on the survival of BBdp rats. Summary of the percentage of animals surviving after saline or NIH injection into both lobes of the thymus at 24h of age. All animals were fed on an open formula rodent diet based on plant proteins (NIH).
Inset shows the qualitative rating ($\bar{x} \pm$ S.E.) of the histology of the pancreas from each treatment, based on a score of 1-5. Normal appearing islets were rated as 1; 2 indicated mild, 3 a majority of islets infiltrated, 4 most islets heavily infiltrated and 5 indicated endstage disease, with few islets, and those appearing to contain only alpha cells, with little inflammatory infiltrate remaining.
* Control HC-fed rats from the first experiment

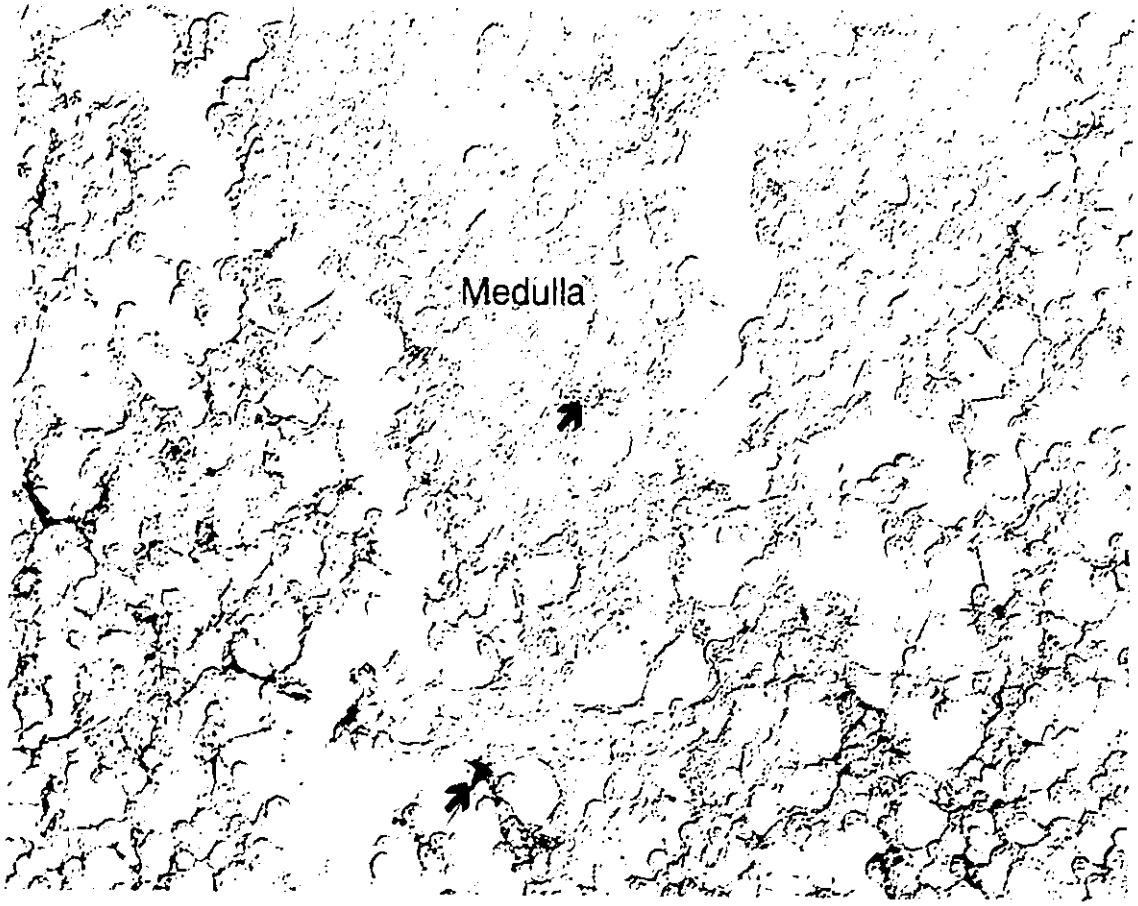

Medulla

Figure 7. Micrograph showing India ink injected into the thymus of rat pup at 24 h of age. Note the India ink (arrows) within the medulla. 720 X. Nomarski optics.

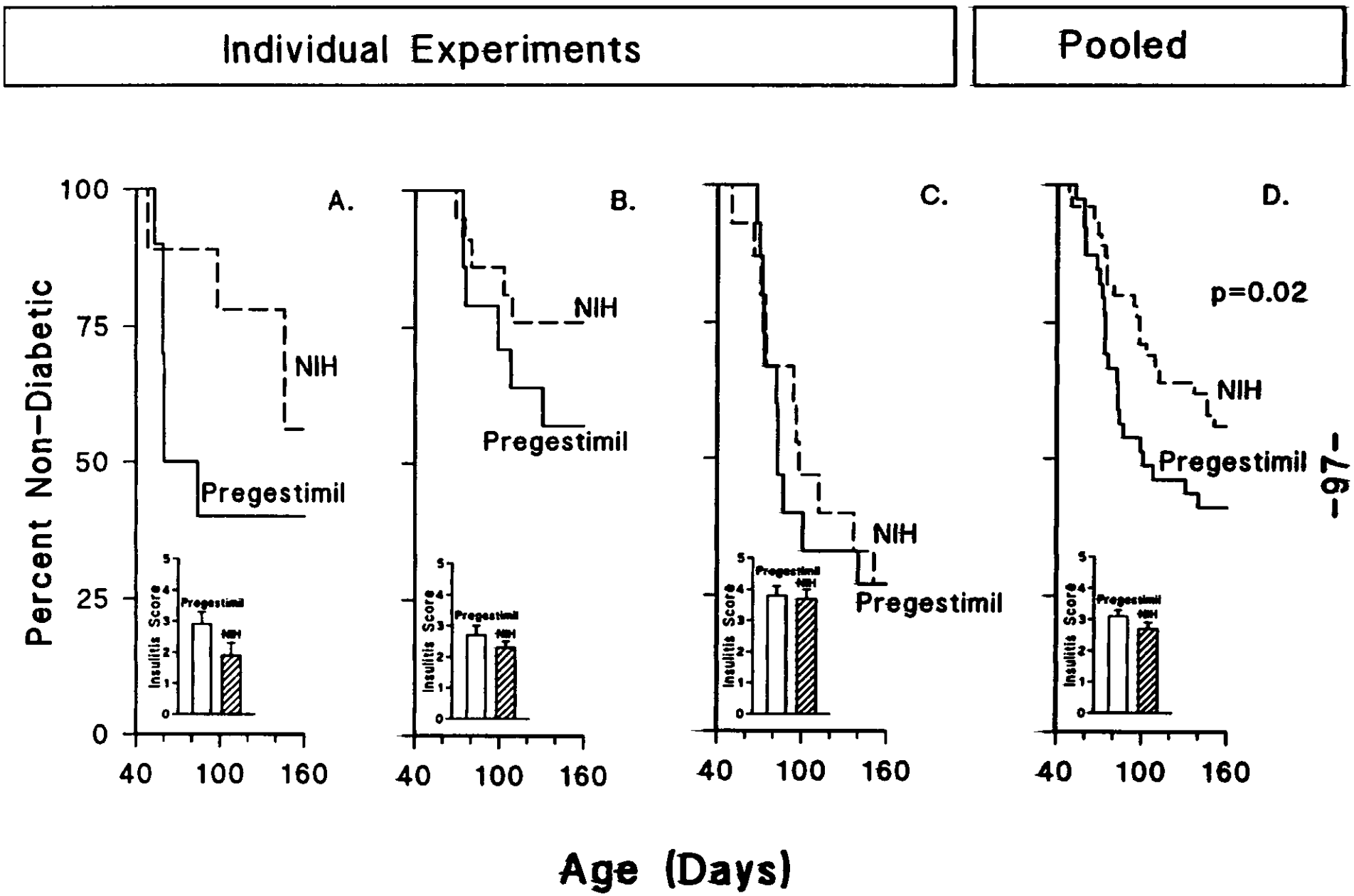

Individual Experiments
Pooled
A.
B.
C.
D.
Percent Non-Diabetic
100
75
50
25
0
NIH
Pregestimil
p=0.02
Insulitis Score
40
100
160
Age (Days)
-97-

Figure 8. Survival of BBdp Rats given NIH as Neonates.
Effect of hand feeding NIH diet to suckling rat pups between 4 and 7 days of life on the survival of diabetes-prone BBdp rats. Rats were weaned onto the diabetogenic NIH-07 (NIH) diet.
Three separate experiments are shown (panel A, B, and C). Inset shows the qualitative rating of the histology of the pancreas ($\bar{x} \pm$ S.E.).
The pooled data from the three experiments are shown in panel D. Significance was calculated by Kaplan-Meier statistics and tested by the log-rank test. Inset shows the pooled qualitative rating of the histology of the pancreas from each treatment, based on a score of 1-5.
A. n=9-10. B. n=14-21. C. n=15. D. n=39-45.

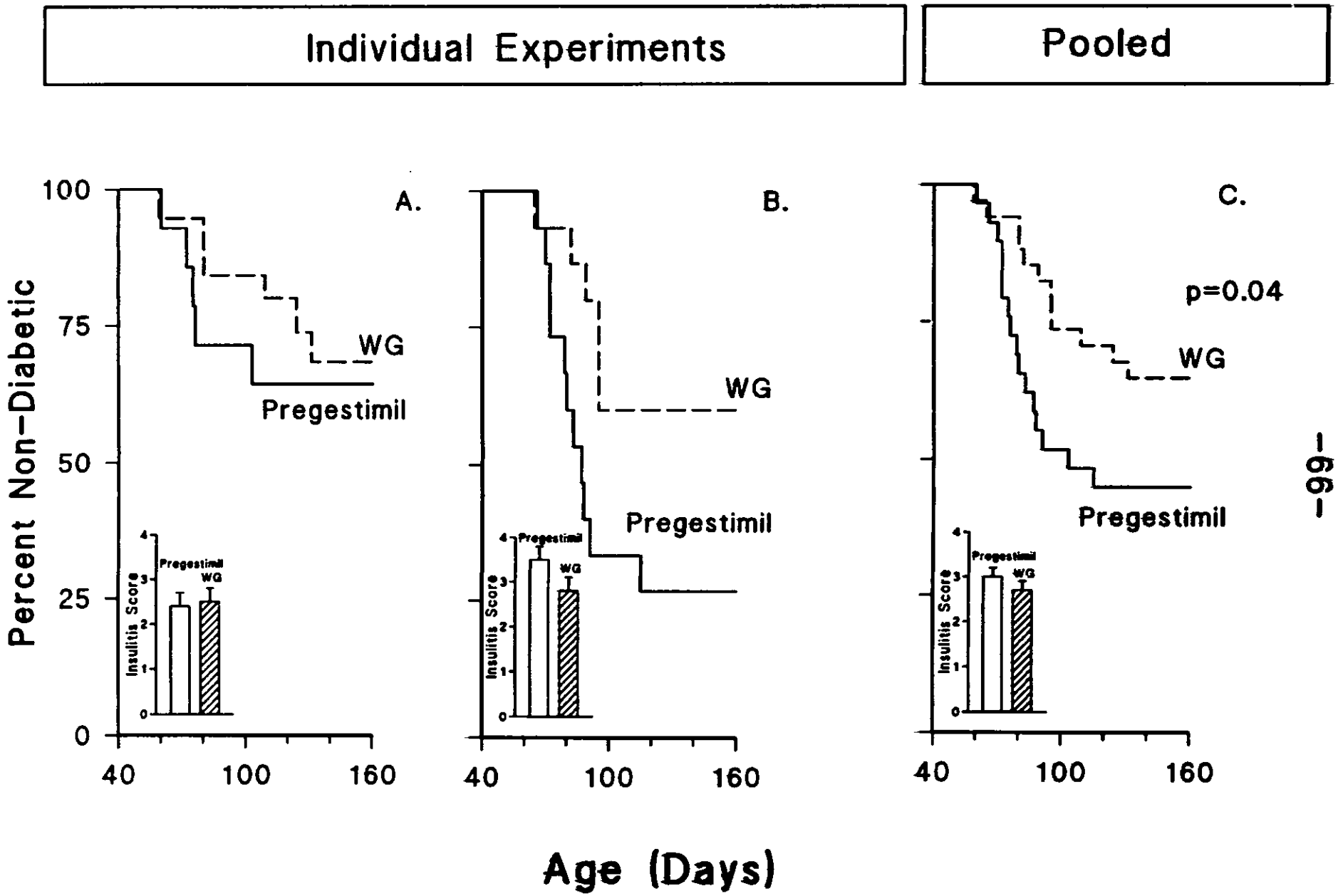
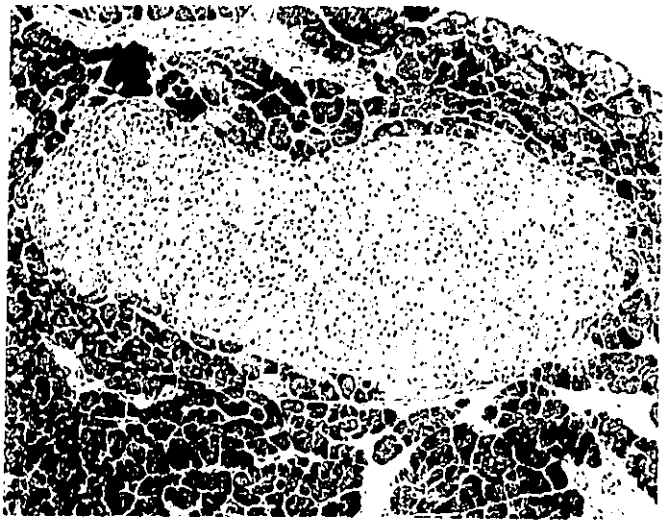
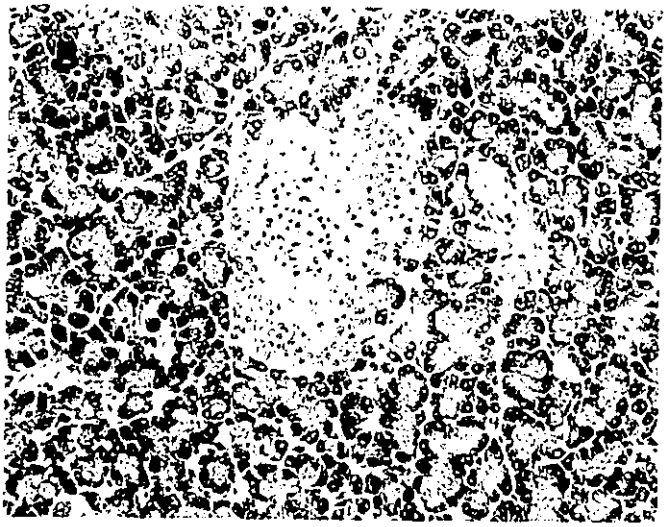
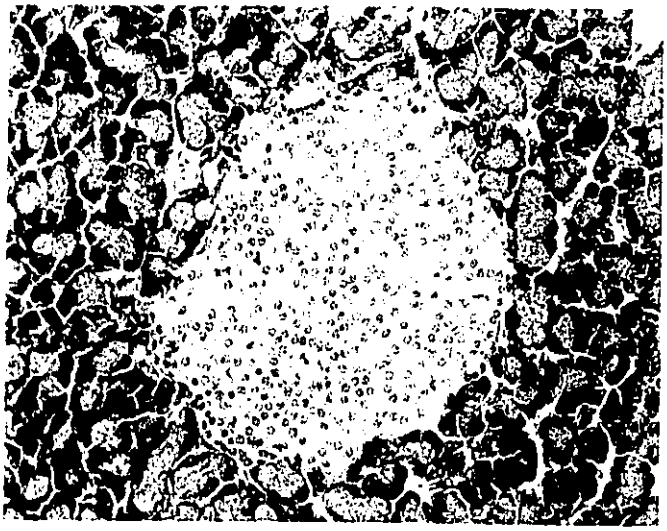

Individual Experiments
Pooled
A.
B.
C.
p=0.04
WG
Pregestimil
Percent Non-Diabetic
100
75
50
25
0
40
100
160
Insulitis Score
Age (Days)

Figure 9. Survival of BBdp Rats given WG as Neonates.
Effect of hand feeding wheat gluten (WG) to suckling rat pups between 4 and 7 days of life on the survival of diabetes-prone BBdp rats. Rats were weaned onto WG based diet.
Two separate experiments are shown (panel A and B). Inset shows the qualitative rating of the histology of the pancreas.
The pooled data from two experiments are shown in panel C. Significance was calculated by Kaplan-Meier statistics and tested by the log-rank test.
Inset shows the pooled qualitative rating of the histology of the pancreas from each treatment, based on a score of 1-5 ($\bar{x} \pm$ S.E.).
A. n=14-19. B. n=15. C. n=29-34.







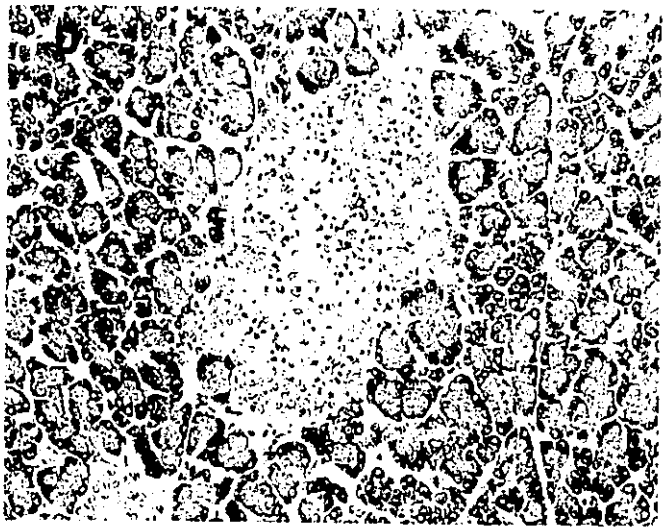


Figure 10. Examples of islets from preweaning treated rats
A. Islet from a rat given wheat gluten (WG) between 4d and 7 d of age and fed a diabetogenic WG based diet. Little insulitis (rated 1.5). 105 X. Hematoxylin and eosin stain (H&E).
B. Infiltrated islet from a rat given the hydolysed casein based infant formula Pregestimil® between 4d and 7 d of age and weaned onto WG based diet. Little of the β cell area remains.210X. H&E.
C. Islet from a rat given the diabetogenic NIH diet between 4 and 7 d of age and weaned onto NIH diet. Little insulitis (rated 1.5). 210 X. H&E.
D. Infiltrated islet from a rat given Pregestimil® between 4 and 7 d of age and weaned onto NIH diet. Little of the β cell area remains. 210X. H&E.

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PUBLICATIONS

Scott, F.W., Cloutier, H. E., Kleemann, R., Woerz-Pagenstert, U., Rowsell, P., Modler, W., and Kolb, H., (1996) Diabetes in the BB rat is mainly food-induced but is prevented by feeding a diabetogen-free diet which increases islet mass and switches infiltrate from Th1 to Th2 cells. Diabetes (submitted).

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ABSTRACTS

Scott, F.W., Rowsell, P. (1995) Controlling diabetes in the BB rat by modifying exposure to food diabetogens: Age at first exposure, dietary dose, and effects of early intrathymic or oral dosing.
(Abstract presented at Diabetes meeting, Rome, Italy, October 1995)

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Scott, F.W., Rowsell, P. (1995) Controlling diabetes in the BB rat by modifying exposure to food diabetogens: Age at first exposure, dietary dose, and effects of early intrathymic or oral dosing. Autoimmunity 21, 21-22.

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