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University of Alberta

Early Diet Modifies Small Intestinal Morphological Characteristics and IgA Bearing Cells in the BB Rat.

by

Shannon Catherine Butler 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

in

Nutrition and Metabolism

Department of Agricultural, Food and Nutritional Science

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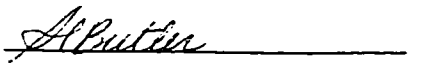

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled *Early Diet Modifies Small Intestinal Morphological Characteristics and IgA Bearing Cells in the BB Rat* submitted by Shannon Catherine Butler in partial fulfillment of the requirements for the degree of **Master of Science** in **Nutrition and Metabolism**.

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Abstract

Type 1 Diabetes Mellitus is characterized by an autoimmune progression of pancreatic destruction and insulin deficiency symptoms. The BioBreeding (BB) rat is a rodent model of Type 1 diabetes. In the present study, non-diabetes prone (BBn) and diabetes prone (BBdp) BB rats were fed either semi-purified casein, a diet associated with low levels of diabetes or standard laboratory chow (NIH-07), known to induce high levels of diabetes. Small intestinal samples were examined at 15 days of age (prior to weaning; pups were housed with chow or casein fed dams) and at 30 days of age (weaned to the same diet as the dam). At 15 days of age, chow fed rats displayed greater jejunal mucosal measures than casein fed rats ($p \leq 0.0001$). At 30 days of age, jejunal mucosal measures in BBn animals were greater for chow fed rats than casein fed rats ($632 \pm 20\mu m$ vs. $501 \pm 9\mu m$, $p \leq 0.0001$). BBdp jejunum did not display significantly altered mucosa in response to dietary treatment. At 30 days of age chow (high diabetes incidence diet) feeding resulted in a threefold increase in jejunal IgA expression in BBdp rats in comparison to casein (low diabetes incidence diet) fed animals ($p \leq 0.006$). Expression of the interleukin 2 receptor was not detected in any diet or strain group. In these experiments we have demonstrated the potential of two diets known to modulate diabetes incidence and onset to modulate intestinal and immune parameters. We have also shown that after weaning, BBdp rats respond to diet in a manner different to weaned BBn rats and those of both strains at 15 days of age. This further indicates the critical changes associated with weaning, and suggests a mechanism by which diet may modulate diabetes incidence.

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List of Abbreviations

APC	Antigen Presenting Cell
BB	BioBreeding
BCA	β-Cell Autoantibody
BBdp	BioBreeding Diabetes Prone
BBn	BioBreeding Normal
BSA	Bovine Serum Albumin
CCK	Cholecystokinin
CD	Cluster of Differentiation
GAD	Glutamic Acid Decarboxylase
GALT	Gut Associated Lymphoid Tissues
GI	Gastrointestinal
GLP	Glucagon-like Peptide
GLUT	Sodium-Independent Glucose Transporter
HSP	Heat shock protein
IA-2	Tyrosine Phosphotase
ICA	Islet Cell Antigen
ICS	Islet Cell Surface
ICSA	Islet Cell Surface Antigen
IEL	Intraepithelial lymphocyte
IFN	Interferon
Ig	Immunoglobulin
IgA	Immunoglobulin A

IL	Interleukin
kDa	Kilodalton
MHC	Major Histocompatibility Complex
NIH-07	National Institute of Health-07
NOD	Non Obese Diabetic mouse
PBS	Phosphate Buffered Saline
SCFA	Short Chain Fatty Acid
SGLT	Sodium-Independent D-Glucose Transporter
TCR	T Cell Receptor
TGF	Transforming Growth Factor
TNF	Tumor Necrosis Factor

Chapter 1: Introduction

The pathogenesis of autoimmune, Type 1 insulin dependent diabetes mellitus remains for clear elucidation. Human Type 1 diabetes is characterized by a rapid progression of the symptoms of polyuria, polydipsia, polyphagia and weight loss in childhood or early adolescence. A hereditary component comprises one of the primary risk factors for this autoimmune condition. A secondary much less defined risk factor is an intricate combination of diabetes susceptibility and environmental influences. This is apparent in the incomplete concordance rates of human diabetes in twin studies (Kyvik et al. 1995, Trucco et al. 1989, Matsuda et al. 1994, Kumar et al. 1993). Factors associated with the etiology of the disease include viral infection, and the introduction of other foreign antigen sources such as diet, vaccine, and chemical agents. Disease 'triggers' are thought to add to β-cell disruption, alter or induce inappropriate immune response and ultimately trigger autoimmunity. The focus of this work will be the association of the prediabetes period and the impact that diet has upon the immune system in a model predisposed to Type 1 Diabetes Mellitus.

Autoimmune diabetes is typically diagnosed at a time when 80-90% of pancreatic β–cells are destroyed and clinical insulin insufficiency symptoms manifest (Virtanen et al. 1994). β-cell destruction occurs in two phases; the first stage is characterized by inflammation, and it is followed by cytolysis of the islet cells (Bach et al. 1995). In both humans and animal models, there are visible histopathological disease symptoms, prior to the presence of overt disease symptoms. The initial infiltration of the pancreas by macrophages, CD4+ T cells, and later CD8+ T cells along with natural killer cells characterize its histopathology in the BB rat (Jiang et al. 1990). Thus, prevention must include early intervention, prior to the development of disease symptoms. Early therapeutic and prophylactic interventions are often aimed modulating immune response and exposure to potential diabetes antigens. Due to the suggested autoimmune pathogenesis of diabetes, interventions that include immunomodulation have and will be considered. Appropriate prophylactic treatments could eliminate the disease-associated disturbances in glucose metabolism, and reduce dependence upon glycemia monitoring, insulin injections, and diabetic complications.

The Etiology of Diabetes: Humoral and Pathogenic Factors

Both in human and animal models of diabetes, the relevance of viral infection in disease pathogenesis has been considered. Viral infection may itself trigger autoimmunity, or a heightened state of immune responsiveness to pathogens may exacerbate a genetic susceptibility to diabetes (Segurado et al. 1993). Autoantibodies could be produced in response to an environmental antigen which has structural similarity to a component of self, in a processed termed molecular mimicry (Schendel et al. 1992, Oldstone et al. 1991, Maclaren et al. 1997). Molecular mimicry may result from exposure to antigens from a variety of sources, including bacteria, viruses, and diet, which would include substances received or produced in the gut (such as microbial peptides in the colon) that have structural similarity to β-cells (Leslie et al. 1994, Maclaren et al. 1997). One of the most popular hypotheses of molecular mimicry is that of the role of viral infection or exposure in diabetes (Rayfield et al. 1987).

The appearance of high levels of autoantibodies in diabetic individuals to self and viral components may be secondary constituents of the disease (Like et al. 1982a, Roep 1996), yet the exact triggers of autoimmunity are not known. β-cell destruction may provide peptide targets for antibody production (that are not found in healthy individuals), particularly β-cell components that are not in native conformations (Nerup et al. 1994). However, despite the presence of pre-disease circulating autoantibodies, only T cells have been shown to transfer the disease in animal models (Birk et al. 1993). Definitive links between the presence of cellular and humoral autoimmunity have not been fully elucidated. Although some autoreactive T cell responses to certain antigens have been associated with the presence of autoantibodies, particularly in the case of insulin (Hummel et al. 1996), the evidence is not overwhelming. The expression of viral antigens on cells infected by viruses may lead to immune mediated destruction of self-cells. Associations of viral infection in humans or human tissue and diabetes have included rubella (Sever et al. 1985), mumps (Prince et al. 1978), cytomegalovirus (Jenson et al. 1980) and Coxsackie B virus (Barrett-Connor 1985). Viral susceptibility combined with genetic disease susceptibility may be associated with increased diabetes incidence. These factors further superimposed upon a background of immune (e.g. limited

suppression or a lack of deletion of autoreactive lymphocytes) or physiological (e.g. a lack of gut wall integrity) susceptibility may further increase both viral and disease risk.

Cellular Immunity and Autoimmunity

Immunosuppressive agents, such as cyclosporine A delay or prevent the onset of diabetes in the BB rat (Laupacis et al. 1983, Like et al. 1984, Jaworski et al. 1986, Yale et al. 1987). Other immunomodulatory agents may also act in similar fashion to alter the incidence and onset of diabetes in this model, including Complete Freund's adjuvant (Sadelain et al. 1990), peritoneal injections of silica (Oschilewski et al. 1985) and 2'deoxycoformycin (Takahashi et al. 1993). This information implies that T cells have a role in the immunopathology of diabetes mellitus. An examination of the role of the cellular immune response in diabetes includes several components; the phenotype of T cells involved, the T cell receptor component (TCR), the antigen presentation component, or major histocompability (MHC) involvement, and the T-helper subset. In humans, MHC class II antigen expression, with respect to HLA markers is a predictive marker of genetic susceptibility to the disease (Li et al. 1995). There is a genetic association of MHC II haplotyping and Type 1 diabetes, as blocking MHC class II interferes with the development of autoimmunity (Rudy et al. 1995). It is suggested that MHC class II expression that occurs on atypical antigen presenting cells (APCs) may trigger or engage self-reactive T cell activation (Durinovic-Bello et al. 1996). As with autoantibody formation, autoreactive T cells may only be a secondary component of the pathology of the disease. However, there are fewer identified autoantigen targets in the T cell population, than there are in the B-cell/autoantibody population (Kallan et al. 1996). The true mechanism of diabetic onset is not known, but it is apparent that they have a place in the disease mechanism.

In humans, potential targets for antigen mimicry (as observed in autoreactive T cell populations) have included a number of pancreatic and cellular targets. The autoantigens most likely recognized by T cells include insulin, GAD65, GAD67, heat shock protein (hsp)65, islet cell antigen 69 (ICA69), as well as β-cell antigens, including insulin secretory granule proteins, and Jun-B, a 38 kDa transcription factor found in the nucleus of β-cells (Bach et al. 1995, Kallan et al. 1996, Roep 1996).

A closer examination of some of these T cell autoantigen targets will elucidate their role in the disease onset. Hsp 65 is a ubiquitous protein, which may have a role as a T cell autoantigen in diabetes, as well as other autoimmune diseases (Roep 1996). Immunity to components of hsp65 is associated with the induction of diabetes in NOD mice; downregulation of hsp65 in this animal model is associated with prevention of autoimmune diabetes (Birk et al. 1993). Another possible autoantigen, GAD65, has been demonstrated to be similar in amino acid sequence to proinsulin (Birk et al. 1993). In a human study investigating the role of cross-reactivity of immune cells to the two antigens, there was increased reactivity in diabetes-susceptible subjects, both as independent antigens, and in combination (Rudy et al. 1995). Proinsulin T cell reactivity was primarily limited to disease-susceptible groups, whereas GAD65 reactivity was spread throughout the control population and those at risk for Type 1 diabetes (Rudy et al. 1995). This suggests a possible role of- proinsulin, and proinsulin-processing as autoimmune targets in Type 1 diabetes susceptible subjects.

The BB Rat

Animal models can provide insightful information and response to diabetes, and its associated pathologies. This may in turn reflect treatment and prophylactic procedures that are effective in the management of human disease. The first and one of the most studied diabetic animal models is the BioBreeding (BB) rat. The spontaneously diabetic syndrome was discovered in Wistar rats in 1970 (Nakhooda et al. 1977) and the line has been further characterized and bred to consist of both diabetic and diabetes-resistant strains (Like et al. 1984). The diabetes prone strain is characterized by diabetic onset between 60 to 100 days, (Butler et al. 1983b, Like et al. 1984) a diabetes incidence of 30 to 90% (Crisa et al. 1992, Pedersen et al. 1994, Baudon et al. 1989) and equal occurrence of disease in both sexes (Butler et al. 1983a, Bach 1988). The diabetic resistant (normal) line has a diabetes incidence of less than 1% (Like et al. 1991). The diabetic prone animals are characterized by lymphopenia (low or absent levels of certain lymphocytes) (Elder et al. 1983, Yale et al. 1985), especially in circulating levels of CD8+ T cells (Poussier et al. 1982, Jackson et al. 1983, Woda et al. 1986), and RT6+ phenotypes (Greiner et al. 1987).

Clinical symptoms of the disease in these animals are characterized by hyperglycemia, weight loss, ketonuria, and hypoinsulinemia (Nakhooda et al. 1983, Like et al. 1984), similar to the human disease. Without insulin treatment, death rapidly ensues. Similar to the human disease, secondary complications including retinopathy (Brown et al. 1983, Cohen et al. 1986, Cohen 35 al. 1987, Chakrabarti et al. 1989), nephropathy (Brown et al. 1983, Cohen et al. 1986, Cohen 35 al. 1987, Chakrabarti et al. 1989), and neuropathy (Sima et al. 1982, Zhang et al. 1990, Sima et al. 1990). Other organ specific sequelae may also manifest in this condition (Meehan et al. 1994, Wright et al. 1983, Miller 1983).

Prophylactic measures, including neonatal thymectomy (Like et al. 1982b) and pre-disease insulin treatment (Gotfredsen et al. 1985, Gottlieb et al. 1991 and Vlahos et al. 1991) protect against disease onset. However, the suitability of diabetic animal models as representatives of the human disease is subject to cautious interpretation with respect to clinical relevance. There appears to exist genetic variability between BB rat colonies (and all animal models) which may lead to discrepancies in testing (Rossini et al. 1995).

Etiology: Humoral and Pathogenic Factors

As in the etiology of human diabetes, viral involvement in disease pathogenesis has been suggested in the diabetes syndrome of the BB rat. The induction of diabetes in BBdp rats can occur when they are infected with Coxsackie B or encephalomycarditis (Berdanier 1995). Kilham rat virus infection can induce diabetes in the BBn rat (Like et al. 1991, Ellerman et la. 1996), and speed the onset of diabetes in BBdp rats (Like et al. 1991, Crisá et al. 1992). The Kilham rat virus is also capable of triggering a diabetes syndrome in RT1 strains that do normally develop diabetes (Ellerman et al. 1996). Thus, though the evidence does not clearly implicate a specific viral agent in the onset of diabetes, its associations may indicate an important role in its pathogenesis.

Possible targets or triggers for autoimmune processes for autoimmune diabetes in the BB rat have included the islet cell surface. Islet cell autoantibodies (ICSA) have been reported (Dyrberg et al. 1982), but their presence remains somewhat controversial (Dyrberg et al. 1984). Other potential targets include a 38kDa β-cell antigen (Ko et al.

1991), glutamic acid decarboxylase (GAD) (Rossini et al. 1995), and carboxypeptidase H (Rossini et al. 1995). Autoantibodies to endothelial cells have also been reported (Doukas et al. 1996), which may be causal to the autoimmune aspects of diabetes or a secondary constituent of the disease. Endothelial destruction could lead to increased vascular permeability, and thus leakage. Vascular leakage proximal to the pancreas could lead to increased systemic exposure of pancreatic antigens, and increase the risk of autoimmunity.

Cellular Immunity and Autoimmunity

The BB rat, and in particular the BBdp animal presents with a number of immune abnormalities that have been linked to the susceptibility to diabetes. BBdp rats exhibit a peripheral T cell lymphopenia that may be characterized by the absence of T cells that express the RT6 surface marker (Crisa et al. 1990, Zadeh et al. 1996). This surface antigen is typically expressed on mature, peripheral CD4+ and CD8+ T cells in the rat and represents a marker of T cell maturation (Greiner et al. 1997, Fangmann et al. 1991). Lymphopenia is present from birth in both diabetic prone rats which do and do not develop diabetes (Yale et al. 1985). Several lines of experimental evidence have indicated that these RT6+ cells (or the absence there of) may be involved in the pathogenesis of diabetes in the BBdp rat (McKeever et al. 1990, Greiner et al. 1987, Jiang et al. 1990). A comparison study of the localization and ontogeny of RT6+ intestinal intraepithelial cells through *in situ* immunohistochemistry (immunoperoxidase staining) in BBn, BBdp and athymic WAG rats (deficient in peripheral RT6 T-lymphocytes) examined the role of thymic development and peripheral migration of RT6 T cells. The study observed that RT6+ intestinal intraepithelial cells appeared before those expressing the αβ-TCR, in all three rat types studied (BBn, BBdp and athymic WAG rats) (Waite et al. 1996). This phenotypic data indicates the role of thymic development; it may have a role in TCR expression, but not in the localization or expression of RT6+ alloantigens in intestinal intraepithelial lymphocytes (Waite et al. 1996). In summary, RT6+ cells migrate to T cell dependent areas in the periphery and RT6+ cells are present in rat intestine before TCR+αβ are detected (Waite et al. 1996).

Despite T cell lymphopenia, BBdp rats appear to have normal levels of B cells

and immunoglobulin levels in lymphoid organs (Elder et al. 1983). However, because of T cell lymphopenia, the proportion of B cells within lymphoid organs is higher (Field et al. 1990, Field 1995).

Along with T cell lymphopenia, lymphocytes from BBdp rats have also been characterized as having altered metabolic activity. Higher utilization levels of glucose and glutamine maybe indicative of some aspect of diabetes pathogenesis (since blocking glutamine metabolism with an analogue, acivicin, prevents diabetes in young BBdp rats, Misra et al. 1996) and lowers mitogenic responses compared to that seen in BBn counterparts. Overall metabolism of immune cells were found to be higher in diabetic BB rats and this was attributed to the increased metabolic activities of the T cells (Field et al. 1994). This difference in metabolism level of BBdp lymphocytes may be indicative of increased immune cell activation (Field et al. 1994). The proposed activation of lymphocytes (primarily T cells) may contribute to the disease-state either through direct actions, or through activation of other autoimmunity-prone immune cells (Field et al. 1994). Increased metabolic activity may then be indicative of immune system functional activity, directed either towards self, or foreign antigens, such as pathogens.

Linked also to immunological activity and diabetes in the BB rat are class II major histocompatibility complex (MHC) antigens. The presence of disease in these animals is dependent upon the heritance of at least one class II $RT1^u$ allele (Colle et al. 1983, Colle et al. 1986, Parfrey et al. 1989). The class II MHC is thought to play a role in clonal selection (Howard 1983), and thus may be involved the generating or failing to halt autoreactive lymphocytes.

The Etiology of Diabetes: Dietary Influence

Human Diabetes and Diet

Among identical twins, the concordance rate of Type 1 Diabetes Mellitus is between 30 and 50% (Kyvik et al. 1995, Trucco et al. 1989, Matsuda et al. 1994); this suggests the role of environmental factors (including diet) in disease onset in accordance with genetic factors. Diet provides an important link between immune function and diabetes for both human and animal models of the disease. Thus, research has examined the mechanisms of dietary influence in disease pathogenesis. This has primarily involved

elucidating specific dietary constituents that trigger autoimmune diabetes processes (Kostraba et al. 1993, Scott 1996) and dietary components that offer disease-protection (LeDoux et al. 1988).

Studies examining infant feeding practices have attempted to determine if a relationship exists between early diet exposure and diabetes prevalence. A number of groups have directed their research efforts toward the earliest non-breast-milk food exposures in infants. Examining cow's milk as a potential diabetes trigger has been an area of increasing interest (Cheung et al. 1994). Further determining a specific antigen within cow's milk as a potential cross-reactive protein candidate for in triggering diabetes has become the focus of several research groups (Cheung et al. 1994, Karjaleinen et al. 1992, Virtanen et al. 1994). This focus has encompassed the study of a specific peptide sequence, ABBOS (part of the BSA molecule) and its relationship to Type 1 diabetes (Martin et al. 1991). There has also been an indicated link between anti-BSA antibodies, BSA-proliferative response of T cells (Cheung et al. 1994), antigen sequence overlap of BSA (Karjalainen et al. 1992) and human diabetes. Other population studies have indicated low or no association between early cow's milk exposure and the presence of β-cell autoantibodies (BCA), which are thought to be indicators of Type 1 diabetes (Norris et al. 1996).

Studies have indicated that early cow's milk exposure in children is not related to increased prevalence of the diabetes-related etiological symptoms (the presence of autoantibodies to glutamic acid decarboxylase (GAD), tyrosine phosphotase (IA-2) (Norris et al. 1996). Despite the apparent association between diet and diabetes, there are currently insufficient long term studies to warrant abolishment of cow's milk feedings in high risk infants (Scott et al. 1996b).

Large scale epidemiological studies analyzing the timing of initial exposure to non-breast milk food sources have found conflicting evidence, although there is a general trend for early cow's milk exposure to be associated with an increased risk of developing diabetes. A meta-analysis of studies that examined the relationship of early introduction of cow's milk into infant diets and short-duration breast feeding by (Gerstein 1994) concluded that patients with insulin-dependent diabetes were more likely to have had

short-duration of breast feeding (less than three months), and an earlier exposure to cow's milk (by four months of age). He concluded that early exposure to cow's milk could increase the risk of type I diabetes by 1.5 times and suggested that removing cow's milk from infant diets could significantly alter the incidence of Type 1 Diabetes Mellitus (Gerstein 1994). Along similar lines, the American Academy of Pediatrics Work Group on Cow's Milk Protein and Diabetes Mellitus formulated a position on the feeding of Type 1 diabetes susceptible infants (i.e., those with a family history of diabetes, or those with siblings with overt diabetes). Their recommendations include breast-feeding of diabetes-susceptible infants, and limited use of casein or soy based infant formulas (Drash et al. 1994).

Epidemiological studies have examined many possible food agents as potential agents to increase the risk of diabetes. Among some of the more common sources are nitrites, and N-nitrosos compounds. These compounds are ingested in association with prepared meats or from drinking water sources. High consumption levels of foods containing nitrates or nitrites have been found to increase the risk of autoimmune diabetes (Virtanen et al. 1994).

Dietary Modulation in Animal Models of Diabetes

The BB rat and NOD mouse represent unique models of diet-sensitive disease susceptibility. Thus, in reference to these models, diets are categorized based on reported effects on the prevalence of diabetes. The two main diet categories include those which have a high diabetogenic potential (such as standard laboratory chow, or NIH-07), and those with low diabetogenic potential (purified diet formulations).

The standard rat chow diet (NIH) is composed of up to 82.5% plant materials (Bieri et al. 1977). The plant material sources vary with soybean meal, ground oats, corn, wheat, alfalfa comprising most of the feed. The non-carbohydrate/plant material portions of the diet include skim milk powder, whey powder, fish meal, and other protein sources. Soybean or corn oils constitute the fat constituent of the diet, and a small proportion, on a weight per weight basis is micronutrient content. This diet has been repeatedly found to be highly diabetogenic in both NOD mice and BB rats (Scott et al. 1985, Hoorfar et al. 1992). When individual components of the diet were tested for diabetogenicity in animal

models, wheat, soybean meal, alfalfa and skim milk powder have exhibited the highest diabetogenic activity (Scott 1994).

Complex or non-purified, mixed cereal based diets (chow) are influential in the onset of diabetes within experimental animals (Ellis et al. 1996). The relationship between insulin required to normalize blood glucose and dietary carbohydrate content (Rajotte et al. 1987) may contribute to the diabetogenicity of mixed cereal diets, such as chow. Diets that induce less insulin secretion, such as purified diets (versus complex diets) may provide an opportunity for β-cell 'rest' (Rajotte et al. 1987) and lower surface antigen expression. A lower level of insulin induction is postulated to be equated to decreased β-cell stress (that is, less insulin production) would reduce insulin-related, or potential β-cell antigens exposure to the immune system (Bach et al. 1995, Scott et al. 1991).

Defined content, semi-purified soy or protein based diets generate lower levels of diabetes than chow (Elliott and Martin 1984, Scott et al. 1985, Hoorfar et al. 1992). However, within purified diets, casein is associated with the lowest incidence and latest onset of diabetes, whereas soy demonstrates intermediary levels between high incidence chow and casein (Hoorfar et al. 1991, Scott et al. 1994). The protective nature of these diets is not fully understood but speculation suggests that either purified diet induces protection in a genetically susceptible model, or carries fewer diabetogens (Scott 1994).

A study completed by Elliot and Martin (1984) fed BB rats semi-synthetic diets substituted with varying protein content: L-amino acids, milk, or wheat protein replacing the natural proteins in the semi-synthetic diet could further alter diabetes onset. Rats fed a standard diabetogenic chow diet and those fed milk proteins had similar diabetes incidence whereas rats fed wheat protein (gliadin) had a lower diabetes incidence. The L-amino acid substituted diet also had a significantly lower diabetic incidence. Thus, protein form (i.e. intake of peptide versus amino acid) and source can affect the development of diabetes (Elliott and Martin 1984) in this animal model. As in human research, milk protein has been included in list of potential diabetogens. However, complete removal of milk protein from diet does not eliminate diabetes in BB rats (Daneman et al. 1987), implicating other factors in the role of disease induction.

Li et al. (1995) found that BBdp rats fed a diabetogenic chow diet, versus those fed a diabetes-retardant (hydrolyzed casein-based) diet demonstrated acute differences in diabetogenesis, as well as MHC antigen expression. Rats fed a chow diet demonstrated increased expression of pancreatic β-cell MHC class I antigen from 25 days of age and expression increased with length of feeding (measurements of MHC expression were initiated at age five days of age). By 50 days of age with chow feeding, hyperexpression of class I antigens was apparent. Approximately 92% of those demonstrating hyperexpression of MHC class I antigens went on to develop severe insulitis and diabetes. In comparison, casein fed rats did not show class I hyperexpression, and these rats failed to develop insulitis or diabetes. Control Wistar-Furth and BBn rats showed only weak background staining for MHC I antigens on their β-islets despite consuming the chow diet. Further examination demonstrated that when chow fed BBdp rats were treated with silica (to inhibit pancreatic macrophage infiltration) hyperexpression of the MHC I antigen was observed particularly due to the increased presence of intact β-cells. There was no detection of MHC class II antigens in any rat type or diet group (Li et al. 1995).

These findings imply a possible role of diet in both immune function and diabetes incidence, yet the actual mechanism of this observed protective effect is not known. There may be a specific component in the diet, or the diet may influence cytokine production and expression, which could in turn modulate MHC expression. Purified diet in BB rats (low diabetogenicity) has also be shown to alter lymphocyte activity, by lowering splenic cytotoxic activity and the decreased metabolism of glutamine and glucose in both the spleen and lymph nodes (Field 1995).

An experiment by Hoorfar et al. (1992) fed BB rats a non-heat treated red lentil-based diet, which demonstrated a diabetes incidence level higher than that of rats fed chow, whereas a diet of 20% (w/w) casein diet was associated with a decreased diabetic incidence. The presence of the red lentil component raised some intriguing implications for disease onset. Red lentils contain lectins that can interfere with gut integrity; and the diet containing the red lentil component was associated with an extremely high incidence of Type 1 diabetes (Hoorfar et al. 1992). This may indicate the role of gut integrity in the

pathology of the disease; impaired integrity of the intestinal mucosa and muscularis may increase systemic exposure to dietary antigens (Hoofar et al. 1992). This increased exposure to dietary antigens could have facilitated increased antigen exposure to the β-cell, which could increase the likelihood of pancreatic immune attack.

Feeding purified soy and casein based diets to NOD mice have also consistently produced low or no diabetic onset (Berdanier 1995). In the presence of purified casein or soy diets (Prosobee and Pregestimil, respectively), nicotinamide further decreases the onset of diabetes and insulitis in the NOD mouse (Reddy et al. 1995), and the addition of 10% bovine serum albumin (w/w) (BSA, an intact protein) to the purified diets did not significantly affect NOD-diabetes onset (Hermitte et al. 1995). BSA is thought to be an antigen that may be related to an autoimmune reaction to β-cells (Cheung et al. 1994, Karjaleinen et al. 1992, Virtanen et al. 1994). However, there are varying reports of autoantibodies in both human populations and experimental diabetes models over the course of diabetes development (Scott 1994).

Intestinal involvement in diabetes pathogenesis is now being examined as a mediator of dietary influence. Reimer et al. (1997) has demonstrated differential expression of intestinal proglucagon with chow feeding between BBn and BBdp rats with greatest expression in BBn rats at 30 days of age. Further, feeding BB rats chow, casein or soy has been shown to alter intestinal parameters, with the greatest expression of colonic proglucagon in chow fed BBn rats (Reimer et al. 1998). The dietary regime also impacted glucose transport capacity; BBn rats displayed the greatest presence of GLUT (sodium-independent glucose transporter)-5 and SGLT (sodium-dependent D-glucose cotransporter)-1 mRNA, above that seen in casein and soy fed rats at 30 days of age.

Dietary nicotinamide has shown some promise as a prophylactic treatment agent (LeDoux et al. 1988). The free radical scavenger may decrease or delay diabetes incidence in both human and animal models. Its efficacy is not fully proven in human diabetes, but clinical trials are underway (Bach et al. 1995). In the NOD mouse model, both the presence of nicotinamide in combination with hydrolyzed casein or soy as the dietary protein source, significantly decreased insulitis and diabetic incidence (Reddy et al. 1995). The protein formulae alone (without nicotinamide) did not suppress insulitis,

but did confer protection from diabetes onset (Reddy et al. 1995).

Dietary modulation of diabetes susceptibility is conditional in that disease modulation is dependent upon age of dietary exposure weaning (Issa-Chergui et al. 1988, Hoorfar et al. 1991). The weaning time point (at approximately 21 days of age) represents a critical point in development, both physiologically and nutritionally (Herbst et al. 1969). Weaning represents a window of susceptibility to dietary influence, as dietary constituents are directed from a liquid, high fat, high protein milk to a solid dietary mixture (low fat, high carbohydrate), without the presence of maternal immunological factors. If the adaptation in diet is to standard laboratory chow, rapid development both immunologically and physiologically must be made to accommodate a high carbohydrate, high fiber, mixed fat and protein diet (Scott et al. 1985). Due to the rapid and extensive nature of adaptation at weaning (Herbst et al. 1969), it is suspected that this is a period where the greatest dietary influences upon development might be seen during this period.

In relation to the potential impact purified diet administration has upon BB rat diabetes, there is a clear pattern linked to weaning. The greatest diabetes-protective affects are apparent when BB rats are fed semi-purified diets at or prior to weaning (Issa-Chergui et al. 1988, Hoorfar et al. 1991). Exposure to cow's milk protein during weaning increases the incidence of diabetes in BBdp rats, whereas exposure to cow's milk protein after 30 days of age does increase the incidence of diabetes (Daneman et al. 1987). The early influences of semi-purified diets have reported to include some protective effects of hydrolyzed casein feeding up to 50 days of age (Scott et al. 1997).

The Gut: A Multi-Functional Organ

The gastrointestinal tract is exposed to a large number of varied antigens into a physiological region responsible to mediating systemic exposure. Not only is it capable of absorbing and distributing required nutrients, it also plays a key role in maintaining a barrier between systemic circulation and exposure to all ingested antigens. It has both immunological and non-immunological protection mechanisms. The non-immunological protection mechanisms include mucosal secretions, peristalsis and rapid epithelial cell turn over (Cerf-Bensussan et al. 1991). The gut has a unique immunological

compartment system, composed of interspersed and concentrated lymphocytic tissues. The intestine itself is frequently referred to as the largest lymphoid organ in the human body (Brandtzaeg 1987). The gut associated lymphoid tissue (GALT) contains immune cells which are spread throughout the villi, lamina propria, lymphoid tissues such as Peyer's patches and scattered lymphoid nodules (Mayer et al. 1996, Weiner 1995, Weiner 1994). M cell-Peyer's patch complexes allow sampling of ingested antigens and act as an antigen surveillance mechanism (Owen 1977, Neutra et al. 1987). M cells and other concentrated areas of lymphoid tissue contain large numbers of antigen presenting cells (APCs) (Bertotto et al. 1991). However, intestinal mucosal epithelium has high levels of expression of class II MHC, which implies the antigen presentation role of non-professional APCs (i.e. epithelial cells) in the gut (Strobel 1996). Presentation of antigen by non-classical or non-professional cells may be indicative of atypical function; either presentation of antigen to cells which are capable of generating clonal responses, or presentation to cells which are not associated with antigen interaction (Mayer et al. 1996).

Early Gastrointestinal Development

As the focus of this literature review pertains to the development of the neonatal BB rat gastrointestinal tract, and in particular the lamina propria region, the known aspects of intestinal development will be addressed. Prior to and including days 14-15 of gestation, cells of lymphoid or myeloid lineage are not present in the rat GI tract (Van Rees et al. 1988a). By day 16 of gestation, the lamina propria contains many cells of dendritic morphology (Van Rees et al. 1988a). At day 18, the lumen of the gut has developed clear definition (Van Rees et al. 1988a). As well, around day 18, the main cell type in the fetal lamina propria is the Ia+ dendritic cell, and some macrophage infiltration can be observed in this region of the intestine (Mayhrhofer et al. 1988). In general, proximal aspects of the small intestine develop more rapidly than the distal aspects (Parrott et al. 1990).

Although studies in rats have examined other time points, neonatal mice demonstrate similar properties of intestinal development (Parrott et al. 1990). Between the age of 10 to 15 days, mice display only scattered IgA plasma cells (Crabbe et al. 1970). Virtually no spontaneous immunoglobulin production is seen in the lamina

propria until three weeks of age (Van der Heijden et al. 1988). By 22 days of age (the weaning time point), there is a large increase in the number of IgA plasma cells, an increase that continues until maturity (Crabbe et al. 1970). Delaying the introduction of solid food (and thus increasing the exposure to maternal milk) delays intestinal immunoglobulin secretion (Van der Heijden et al. 1990). Whether solid diet provides antigens that induce IgA production at weaning, or maternal milk contains factors which downregulate the production of secretory immunoglobulin is not clear.

Another important consideration with respect to the neonatal rat model and its intestinal tract is the concept of gut closure. This developmental stage signifies that the permeability of the mucosal barrier of the gut has reached its mature status. Absorption of immunoglobulin across the jejunal epithelium (closure) occurs until approximately 21 days of age in rats (Brambell 1970). Suckling rats demonstrate an increased rate of macromolecular transport across the small intestinal epithelium at the time of gut closure, when concurrently exposed to dietary antigens (Isolauri 1993). Exposure to cow's milk antigens prior to gut closure can increase their absorption across the villus epithelium (Isolauri 1993). The early 'leakiness' of the gut, that is, prior to closure may also allow antigen exposure to an underdeveloped gastrointestinal immune structure (Walker 1974) and potentially trigger inappropriate or early activation of immune responses. Administering exogenous IFN-γ prior to gut closure was shown to increase the uptake of intact horseradish peroxidase without affecting post-closure uptake (Sutas et al. 1997).

Even after closure, antigen exposure may continue through transcellular means (endocytosis), paracellular entry, or through M-cell/Peyer's patch antigen uptake (Brandtzaeg 1996). The context of immune response to dietary and environmental antigens is then governed by the gastrointestinal immune control mechanisms at the level of organized immunological structures, such as the Peyer's patches and other less differentiated tissues (Brandtzaeg 1996). Understanding the development of tolerance mechanisms is important in the neonatal human and animal. Newborn antigen exposure occurs either through direct oral administration, or indirectly, through diet (breast milk). Breast milk may provide protection to the underdeveloped newborn intestinal tract from antigen sources; the concentrations of antigens present in breast milk are significantly

lower than those found in the maternal diet in both humans and rodents (Axelsson et al. 1986, Troncone et al. 1988). Low, repeated antigen exposure, associated with breast milk may suppress immune responses, whereas oral antigens administered during the neonatal period from non-breast-milk foods may sensitize as opposed to tolerize an individual (Strobel 1986). If animals are fed non-breast-milk foods during the earliest days of life, these foods may prime the immune system for subsequent immune responses and antigen exposures. This may be important as experimental evidence in rodents demonstrates the first fourteen days of life as a time for rapid change in gut mucosa and systemic immune response to oral antigens (Strobel 1986).

At weaning, there is short disruption in the ability of which tolerance can be induced in the young rodent. There is also a decrease in systemic tolerance during weaning (Strobel 1996). Morphologically, the gut at weaning is characterized by a lengthening of the crypts and, increased numbers of intraepithelial lymphocytes, mucosal mast cells, eosinophils, and jejunal goblet cells, as well as increased epithelial cell turnover (Parrott et al. 1990). These changes in morphology may be indicative of the changing challenges placed on the gut; a switch from breast milk to solid foods or other food sources will increase antigen exposure and this will require a greater capacity for an independent immune response. In adult mice, oral tolerance is apparently induced most commonly with soluble antigens; however, the effect of insoluble protein antigens on tolerance induction is not fully known (Strobel 1996). Responses in neonatal mice to antigen before and at weaning (a tentative age point, which can define the end of neonatal life) represent different aspects of oral tolerance (Strobel 1996). Antigen exposure or feeding at an early time point (that is, during the first day or two of life), followed by another oral challenge at four weeks of age, is associated with a demonstrable increase in intraepithelial lymphocytes and crypt cell production, events compatible with cell mediated immune response (Strobel 1986). Based on experimental evidence, and intestinal morphological adaptation, it is apparent that antigen exposure in rodents, five to seven days after birth, decreases the likelihood of tolerance induction (i.e. reduced suppression of cell mediated immune response to the antigen); whereas later exposures are more likely to favor the humoral responses which are associated with tolerance

(Strobel 1996).

Antigens that pass through the gastrointestinal tract are subject to many agents that may alter their structure and antigencity before absorption or interaction in immune response. Prior to absorption, antigens are exposed to the actions of the secretory products of the stomach, pancreas, and bacterial products throughout the gastrointestinal tract (Mayer et al. 1996). However, the gut does allow the passage of some ingested antigen, typically intact protein that bypasses complete digestion, into systemic circulation (Brandtzaeg et al. 1987). Whether tolerance is generated through gut exposure, or simply because the antigen reaches the systemic immune system, and is recognized as a 'self' component is not clear (Friedman 1996). One theory suggests that soluble antigens are taken up by intestinal epithelial cells, and elicit either an anergenic or tolerant response, depending on the presence of other signals (Mayer et al. 1996). Insoluble antigens (such as in immune/antigen complexes) are more likely to be taken up by intestinal M cells, or dome epithelial cells, which in turn evoke a secretory IgA response (Mayer et al. 1996). Inducing tolerance of the gut in young animals may decrease the risk of autoimmune cross reactivity with environmental antigens. Further, it may decrease the risk of premature immunological development.

Intraepithelial Lymphocytes

Interspersed throughout the epithelium are intraepithelial lymphocytes (IEL) which are located predominantly along the basement membrane (Marsh 1975). Most of these lymphocytes bear the CD3+ phenotype, whereas lamina propria lymphocytes are predominantly CD3+CD4+ (Lefrancois 1991). In humans, the TCR type associated with IELs is typically $\alpha\beta$, much fewer are of the $\gamma\delta$ form, whereas rodents express higher levels of the $\gamma\delta$ TCR (Jarry et al. 1990, Van Kerckhove et al. 1992). IELs are important in the defense mechanisms of the gut since they help to limit pathogenic infection through direct cytotoxic effects and cytokine expression, particularly in the form of IL-2, IFN-γ and TNF-α (Taguchi et al. 1990). In the induction of oral tolerance, IELs may act in a suppressive capacity, either through the production of soluble mediators (cytokines) or other mechanisms (Cerf-Bensussan et al. 1991).

Lamina Propria

The immune tissues underlying the epithelium of the gastrointestinal tract are those of the lamina propria. Usually located as dispersed immune cells, lamina propria lymphocytes are approximately 20-40% B cells (Beagley et al. 1992). In adult rats, mice and humans, most of the T cell populations of the lamina propria are CD4+ (Parrott 1987) and express the αβ TCR (Beagley et al. 1992). Immunocytes within the lamina propria express markers of activation, including the IL-2 receptor (Schieferdecker et al. 1992, Zeitz et al. 1988).

Peyer's Patches

The organized lymphoid tissues of the intestinal mucosa, Peyer's patches, function as areas for antigen sampling, T cell response suppression and the induction of IgA responses (Heel et al. 1997). Peyer's patches are found predominantly at the ileum (Wolf et al. 1981). Exposure to dietary and environmental antigens is mediated primarily through these 'windows' within the intestine (Challocombe 1995). Peyer's patches contain the majority of B-cell (precursors plasma cells) which are ultimately the source of the IgA producing and secreting immune cell population found in intestinal mucosa (Mestecky et al. 1989, Mestecky 1987). B-cells eventually localize in the lamina propria, where they ultimately differentiate and proliferate (Brandtzaeg et al. 1987). Peyer's patches also contain T cells, which are predominantly of the TCR αβ form, with the majority expressing CD4+ (Mestecky et al. 1987).

Humoral Intestinal Immunity: The Spotlight on IgA

A cellular immune response within the gut associated immune system is essential for developing oral tolerance but humoral responses also play an important role in this immune capacity of the gastrointestinal tract. The humoral response is generally under the control of T cell regulation; T cell help is required for the appropriate activation and production of immunoglobulins (Mestecky 1988). The gut represents the largest region of IgA production and secretion in humans (Mestecky et al. 1987, Seilles et al. 1985). The greatest production of IgA occurs in the plasma cell-rich lamina propria and Peyer's Patch regions of human intestinal mucosa (Beagley et al. 1992, Mesteckey et al. 1987). IgA exists in the gut in a secretory form, primarily as a dimer with an associated secretory

component, necessary for secretion into the lumen of the gut, and a 15 kDa J chain, important for its secretory chain binding site (and polymerization). After its production, IgA is externalized via its secretory component (polymeric immunoglobulin receptor) (Brandtzaeg et al. 1992) via ligand/receptor means. In rodents, the secretory component is produced by crypt epithelial cells and by hepatocytes, although it is not normally expressed prior to 20 days of age (Vaerman et al. 1989).

IgA production is triggered through a primarily Th2 cytokine cascade, which allows for immunoglobulin class switching and clonal expansion of B cells (Xu-Amano et al. 1992). Transforming growth factor-β, IL-2, and IL-4 are crucial in the early events leading up to IgA expression. IL-5, IL-6 and IL-10 are important in the latter stages of IgA production, inducing B cell proliferation and IgA-J chain production and secretion (Brandtzaeg 1996). Thus, T cells located in the surrounding mucosa are capable of regulation and modulating of IgA responses and hyporesponsivness (Kiyono 1982).

IgA is not related to the inflammatory response, as other immunoglobulin subtypes are since it does not have a role in complement activation or other inflammatory elements (Brandtzaeg et al. 1977, Russell et al. 1989). Secretory IgA functions specifically to disrupt bacterial invasion and adherence, neutralizes toxins, agglutinates bacteria and inactivates enzymes involved in virulence (Childers et al. 1989). Its function may not be limited to the lumenal portion of the gut since its presence on the basolateral portion of the gut may act to contain antigens that have made it through the enterocyte barrier at the lamina propria (Brandtzaeg 1996) or through M cells (Kaetzel et al. 1991, Mazanec et al. 1992). IgA may also act internally within enterocytes to prevent the passage of viral particles (Mazanec et al. 1995, Mazanec et al. 1992). Within the human gut, IgA subtypes dominate particular regions: IgA1 predominates in the upper GI tract, IgA2 the lower tract, including the colon (Kett et al. 1986). The ratio of IgA1 to IgA2 varies between the lower and upper gut, and may reflect the functionality of the immunoglobulin subset (Mestecky et al. 1986). IgA2 is thought to be more resistant to bacterial IgA proteases, and hence predominates in the large intestine (Plaut 1983). Within the rat, only one isoform of IgA has been characterized (Nash et al. 1969, Stechschulte et al. 1970).

In the adult human, the greatest production of IgA occurs in the gastrointestinal tract (up to 80%) (Brandtzaeg 1985). In other mammals, including the rat, there is comparably less gastrointestinal IgA production. In rats, IgA production is predominately hepatobiliary (Manning et al. 1984), and along with circulating IgA (which is actively transported from blood to bile at the liver) and is ultimately secreted in bile (Ahnen et al. 1985). Thus, rat bile is a rich source of IgA (Fisher et al. 1979). Within the GI tract, IgA production occurs predominately in the lamina propria, which contains large numbers of B cells, plasma cells, T cells, macrophages and antigen presenting cells (Mowat et al. 1997).

IgA acts to decrease exposure to infectious agents, and other antigens. These two factors are critical both in early intestinal development and disease prone states which may trigger autoimmune processes. The absence of IgA secretion/production has been associated with a wide range of clinical conditions, from very mild local symptoms to associations with autoimmune disease (Liblau et al. 1992) including autoimmune diabetes (Smith et al. 1982, Hoddinott et al. 1982). IgA deficiency is the most common primary immunodeficiency in the human population (Frommel et al. 1973, Koistinen 1975). The deficiency syndrome, further exemplifying the function of IgA, is often characterized by recurrent gastrointestinal and respiratory tract infections (Ammann et al. 1971), allergies (Plebani et al. 1986), but in some individuals may remain asymptomatic (Koistinen 1975). Still other reports suggest an increased presence of IgA in autoimmune disease. There are reports of increased levels of IgA in the serum of diabetic populations (Rodrigeuz-Segade et al. 1996), and at the time of diabetes onset (Gorus et al. 1998).

Several factors have been postulated to alter the production and secretion of IgA within the gastrointestinal tract in the rodent. Intestinal microflora has been demonstrated to alter sIgA presence in a rodent model (Crabbe et al. 1968). Altering intestinal flora with the administration of an oral *Saccharomyces* species increases intestinal production of IgA and its secretory component (Buts et al. 1990). Mice raised in germ-free conditions demonstrate little or no IgA production in comparison to conventionally housed mice (Crabbe et al. 1968). Thus, the antigenic stimulus provided by changing the intestinal flora may ultimately alter immunocyte population development (Crabbe et al.

1968). Further, diet has been shown to alter intestinal flora (Gibson et al. 1995), which reinforces the potential for diet to modulate intestinal immune function.

Isolated, adult rat intestinal loops subject to prior antigenic stimulation demonstrate a dose dependent response in IgA production to intravenous cholecystokinin (CCK) administration (Freier et al. 1987). The mechanism of enhanced IgA production is not fully elucidated, but has been suggested as involving several of the following potential mechanisms. Increases in motility in the small intestine due to CCK stimulus may increase the release of preformed IgA, or CCK may act in a more direct manner to signal immunoglobulin release at the level of the lamina propria (Freier et al. 1987). Substrance P, as well as feeding (through CCK) can also increase IgA release; cholinergic antagonists can inhibit IgA release in rats (Freier et al. 1989).

Interleukin 2 and its Receptor

A large number of immunological factors interact at the intestine in a variety of capacities to ensure immune defense and regulate cell populations. Indeed, many of these factors can alter facets of intestinal function. In the expanding field of cytokines and chemokines many mechanisms of action remain to be established. Interleukin 2 (IL-2), and its receptor (IL-2R) comprise a critical component of GALT, functioning in multiple roles, including oral tolerance (Dahlman-Hoglund et al. 1996) and IgA synthesis (Brandtzaeg 1996). IL-2 is classified as a lymphotrophic or stimulatory cytokine. Produced by activated T cells responding to the appropriately processed and presented antigen, IL-2 and acts to stimulate lymphocyte activation and proliferation (Baker et al. 1978, Larsson et al. 1980, Smith et al. 1980). IL-2 then regulates a number of the activities of T cells, B cells, natural killer cells, and monocytes (reviewed in Greene and Leonard 1986, Waldmann 1991).

The IL-2R is composed of three subunits, the α,β, and γ chains (Miyajima et al. 1992, Smith 1988, Bazan 1992). These subunits of the receptor are able to combine in several conformations, of varying affinity. These combinations involve the α chain form (low affinity), $\beta\gamma$ (moderate affinity), and the high affinity formation, $\alpha\beta\gamma$ (Waldmann 1993, Bazan 1992). The γ subunit is constitutively expressed, while the α and β chains are expressed only upon activation. Activated T-cells express both the high and low

affinity IL-2R subunit combinations (Takeshita et al. 1992).

There is a suggestion that altered IL-2 production, or its receptor may be associated with human autoimmune diabetes (Giordano et al. 1989, Zier et al. 1984). Reduced IL-2 secretion has also been reported in BB rats (Prud'homme et al. 1984). Exogenous IL-2 administration to BBn rats results in an increased prevalence of diabetes (Kolb et al. 1986), and reduced diabetes incidence in BBdp rats (Zielasek et al. 1990). Thus, although not clear, there may be IL-2 involvement in Type 1 diabetes pathogenesis. Or alternatively, abnormalities its expression or production may be reflected in immune activation in the disease process.

As the IL-2 receptor is expressed solely upon activated lymphocytes, its presence is an indicator of immune activation and response. Within the gastrointestinal tract, the expression of IL-2R is not limited to lymphocytes; it may also be expressed upon intestinal epithelial cells (Ciacci et al. 1993). Its presence at the epithelium has been postulated to be important in the maintenance of the epithelial barrier (Diagnass et al. 1996). IL-2R expression at the lamina propria of the intestine in rats is suggested to maintain tolerance to orally introduced antigens (Dahlman-Hoglund et al. 1996). Again, the full functional roles of the IL-2 receptor and ligand or other potential ligands has not been fully elucidated, but a potential function in epithelial barrier function (Diagnass et al. 1996) and oral tolerance maintenance (Dahlman-Hoglund et al. 1996) has been presented.

Oral Tolerance

Oral tolerance is the mechanism by which antigens are ingested, and subsequent parenteral challenges with the same antigen demonstrate a hyporesponsive immune reaction (Weiner 1994). This tolerance may be party to one of two main tolerance hypotheses; the first of which suggests that tolerance is a result of a suppression or tolerance that is actively induced through the immune system, or, secondly, tolerance is a product of the barrier provided by the gut, through the mucosa and tight junctions, such that antigen access to the systemic circulation is prevented (Mayer et al. 1996). Regardless, the mechanism is subject to a combination of a number of factors, which include exposure to antigen type, quantity of antigen in exposures, host status, and the

frequency, age of first exposure to the antigen (Strobel 1996) and genotype of the host (Vaz et al. 1987, Lamont et al. 1988). However, it is apparent that tolerance is induced through active suppression through the immune system (Friedman 1996).

There are three main mechanisms through which tolerance is induced both in the gut and the rest of the body; clonal deletion, clonal anergy and cytokine mediated suppression (Friedman 1996). Oral tolerance is dependent upon both the type and amount of antigen presented. There is a relationship between antigen dose, and clonal anergy (Weiner 1994). Very high doses of antigen may be associated with clonal deletion (Weiner 1995, Weiner 1994, Strobel 1996). These types of responses are not mutually exclusive; some antigens may induce both suppressive and anergenic effects (Miller et al. 1992). A suppressive reaction is characterized by T cell mediated secretion of immunosuppressive cytokines, including IL-4, IL-10, and TGF-β (Powrie et al 1993, Romagnani 1992). Clonal anergy responses are typified by the anergy of Th1 components, including decreases in IgG2a, IL-2, and IFN-γ, and maintaining the capacity for Th2 response, which is characterized by the presence of IL-4 and IgG1 (Friedman 1996). In rodent models, a high dose antigen exposure is defined as >1mg/g body weight and a low dose exposure is associated with a >0.1mg/g body weight administration of antigen (Strobel 1996).

Gut tolerance may hold a key in the prevention of Type 1 diabetes; the presence of pancreatic autoantibodies indicate multiple autoantigen targets (Maron et al. 1996). If tolerance can be induced to β-cell antigens, the disease could potentially be halted. Yet determining which antigens can be targeted to arrest pancreatic destruction remains difficult. A concept termed bystander suppression (Miller et al. 1991) may provide both an answer, and therapeutic potential in several autoimmune diseases (Weiner et al. 1994, Amital et al. 1996), including diabetes (Muir et al. 1993). The concept of bystander suppression suggests that effector cells that regulate immune response are introduced to oral antigen at the gut, these effectors develop a suppressive cytokine response to certain antigens, and home through systemic circulation to other regions (Miller et al. 1991). Further, if the effectors reside at an area of inflammatory response in an organ environment where the initial antigen is localized, their suppressive cytokine response

could ultimately reduce inflammatory response (Miller et al. 1991).

Generating bystander suppression, or tolerance would be characterized by the local effects of the tolerance response, including activation of Th2 cytokines, and TGF-β (Chen et al. 1994). Thus, generating oral tolerance to a known autoantigen in the disease, like insulin, through repeated low dose exposures, might provide the organ specific protection required for the prevention of the disease (Weiner 1995, Weiner 1994). Studies in NOD mice have indicated that oral insulin administration can lead to the development of a suppressive response to insulin (Bergerot et al. 1996). However, the amount of insulin (or antigen) delivered is paramount in developing tolerance (Bergerot et al. 1996). Insulin is not the sole candidate in this search for antigens that may provide tolerance; the administration of GAD may also be effective, or other islet cell proteins could possibly act to provide bystander suppressive effects to the pancreas (Bach et al. 1995). Oral tolerance induction to diabetes or pancreatic-related peptides has been reported in NOD mice (Maron et al. 1996), but not in the BB rat (Mordes et al. 1996). In the rat, the liver is important for developing oral tolerance; microbial and food antigens that reach the liver via portal circulation can reduce systemic responsive to the antigen, and Kupffer cells play an important role in inducing suppression of immune responses (Thomas et al. 1976).

Tolerance is not limited to a potential therapeutic application for autoimmune conditions, it is also important in food allergy. The gastrointestinal tract plays a key role in mediating systemic antigen exposure (Cerf-Bensussan et al. 1991, Brandtzaeg 1996). Systemic exposure to antigen, specifically in the neonate, is associated with increased antigen exposure and potential sensitization to antigens (Host 1994, Strobel 1986). As allergy and tolerance are regulated by immunological mechanisms, a relationship between abrogated tolerance and sensitization may occur in the case of food allergy; potentially at the level of the gastrointestinal tract. In neonates, this commonly translates into the presence of cow's milk allergy (Hill et al. 1995, Docena et al. 1996), associated with early exposure to cow's milk protein allergens (Host 1994). The allergic response is controlled in a manner similar to tolerance induction; that is via a Th2 cytokine profile (IL-4, IL-5). However, the presence of IL-2, IgE release, histamine release and eosinophil

degranulation (Hauer et al. 1997, van Halteren AG et al. 1997) differentiate it from tolerance. Thus, inducing tolerance or systemic exposure may ultimately prevent the induction allergy.

The γδ effector cell is contained in relatively high concentrations within the epithelium of mucosal surfaces (Goodman et al. 1988) predominantly as intraepithelial lymphocytes (IELs). The lamina propria has greater numbers of γδ T cells than any other systemic tissue (Aicher et al. 1992), which may implicate their role in gut immune function, but the actual role of these T cells is not fully known. γδ knockout mice exhibit immune abnormalities namely, decreased IgA production, suggesting a possible role of γδ-cells in regulation of the IgA B-cell response (Fujihashi et al. 1996). Speculated actions for γδ T cells include early host mucosal defense against pathogens, or involvement with antigens expressed on stressed epithelial cells (Kagnoff 1993). The association of γδ T cells with IgA production, which is a primary Th2 cytokine driven process, has also been speculated to influence oral tolerance induction (Fujihashi et al. 1996).

Diet and Intestinal Modulation

As suggested in previous segments of this review, diet has the capacity to impact a number of facets of disease and immune function. This section will further examine the potential for diet to modulate intestinal structure and intestinal immune function, with specific attention to the neonate. At birth, the intestine is essentially sterile (Rognum et al. 1992); its first exposure to antigen occurs in the form of milk. Thus, the neonate is exposed to changing diet via alterations in maternal diet, which are reflected in changes of the composition of maternal milk (Brandorf 1980, Read et al. 1965). Dietary antigens presented at the gastrointestinal tract may then be acted upon in different manners; altered cytokine and immune cell content, such as those in maternal milk (Noda et al. 1984, Munoz et al. 1990, Saito et al. 1991, Bocci et al. 1993), have the capacity to impact antigen uptake through GALT (Sutas et al. 1997). The rate of antigen uptake can further influence the disposition of the intestine to inflammation (Heyman et al. 1987).

Diet may have another essential connection to immune function. The presence of colonic microflora is required for the development of the ability to produce IgA (Crabbe

et al. 1968). Although somewhat controversial, alterations in colonic microflora may occur in response to dietary intake (Swords et al. 1993, Gibson et al. 1995). Thus, foods in the diet may have the capacity to directly or indirectly alter IgA production and potentially gastrointestinal immune function. Studies in the BBdp rat, have demonstrated the ability of dietary constituents to modify intestinal immunocyte cytokine mRNA production (Kleeman et al. 1998), further reinforcing that diet can alter intestinal immune function. Peripheral immune cell populations have been demonstrated to be altered with semi-purified casein and soy feeding in BB rats (Field (a) et al. submitted).

Immune function and GALT can be influenced directly by dietary manipulation. In weanling rats, dietary protein-malnutrition decreases the presence if IgA in small intestinal fluids (Sullivan et al. 1993) and in the intestinal lavage contents of mice fed protein-deficient diets (McGee et al. 1988). IgA levels have been further suggested to be influenced by reduced expression of the polymeric immunoglobulin receptor at the intestine and liver, as described in mice fed protein-deficient diets (Ha et al. 1997). In humans, experimental evidence has examined the impact of infant feeding practices upon IgA. Formula fed infants display an earlier and higher level of salivary IgA in comparison to breast fed infants (Gleeson et al. 1986). Infants fed breast milk and cow's milk formula had salivary IgA levels lower than formula fed infants, but relatively higher IgA levels than infants exclusively fed breast milk (Gleeson et al. 1986). Clearly, diet can affect IgA levels at the intestine in the case of protein malnutrition, however, the affects of other specific dietary constituents or less severe changes in nutrient intake upon immunoglobulin production have not yet been elucidated (Ferguson 1994). Dietary fiber has been reported as influential at GALT as increasing the fermentable fiber in dogs is able to alter T cell mitogen responses throughout GALT, and CD4+/CD8+ ratios (Field (b) et al. submitted). Other nutrients may also impact various components of GALT function (Hangkamp-Henken et al. 1992).

Just as diet has the potential to modify GALT, a large number of factors may influence intestinal structural and functional development. Diet has been shown to modulate the release of human gut hormones (Lucas et al. 1980). Thus, diet-induced changes gut hormones could have a potential impact upon immune tissues, reflected in

altered antigen uptake, and structural and functional modifications. A number of factors working in conjunction with nutritional constituents, or alone, may influence intestinal adaptation and trophism. These may include epidermal growth factor (EGF) (Goodlad et al. 1985, Gasslander et al. 1997), glicentin (Myojo et al. 1997), insulin-like growth factor (IGF)-1 (Zeigler et al. 1996), and glucagon-like peptide 2 (GLP-2) (Tappenden and McBurney 1998, Tappenden et al. 1998).

Dietary effects, such as those seen in work by Reimer et al. (1998), demonstrate changes in glucose transport capacity in response to feeding casein and chow diets in BBn and BBdp rats. They concluded that this may reflect differences in the ability of the gut to adapt to changes in nutrient intake (Reimer et al. 1998). Trophic factors, as listed above, may alter absorption, via increases in mucosal surface area, including epithelial surface or increased glucose transporter expression and activity (Ferraris et al. 1997). Reimer et al. (1998), has indicated an increased expression of intestinal proglucagon in chow fed BBn rats compared BBdp rats at 30 days of age. This change may be the result of altered capacity of weaned BBdp rats to adapt to the presence of complex carbohydrate in diet. The presence of increased proglucagon message is associated with increased postprandial secretion of GLP-1 and insulin (Reimer et al. 1997, Massimino et al. in press) and increased intestinal glucose transport capacity or expression of glucose transporters which have sometimes been associated with elevated plasma GLP-2 concentrations (Reimer et al. 1998, Massimino et al. in press, Tappenden and McBurney 1998, Tappenden et al. 1998). GLP-1 is a known insulin secretagogue (Holst 1994) and may mediate small intestinal glucose transport (Cheeseman and Tsang 1996). Thus, many factors have the potential to influence intestinal structure, GALT function and intestinal function in the BB rat and other models.

Conclusion

Genetic factors are well established as altering the risk of developing Type 1 diabetes, but heredity is not the sole factor in diabetes susceptibility (Kyvik et al. 1995, Trucco et al. 1989, Matsuda et al. 1994, Kumar et al. 1993). Many other environmental components have been implicated as being involved in the etiology of diabetes. These risk factors may be grouped into several categories; those directly associated with

initiation of the disease, and those associated with its promotion in susceptible individuals. Initiators of the disease are considered to be those components that specifically induce β-cell destruction. Those factors, which may promote diabetes, may be diet associated, and from human studies include short-duration or limited breast feeding, and factors which could generate β-cell stress. Questions remain as to the biological latency period of the disease, and the windows of time in which susceptibility to the disease exists, in both human and rat models (Drash et al. 1994).

Type 1 diabetes is a disease of multi-factorial etiology, symptoms, and complications. Modulation of the autoimmune mechanisms in the rodent models can be accomplished through dietary regulation. This suggests a role for the gastrointestinal tract, and its immune tissues. The BB rat provides a valuable model, with similarities to the human disease, and it is particularly useful as a first line for examination of the etiology of autoimmune diabetes, its prophylaxis and possible treatments. Future considerations for the treatment of diabetes may be based on the development and application of prophylactic measures as well as disease control modalities. Clearly the BB rat presents a case for maternal dietary prophylaxis, dietary intervention in early life and oral tolerance to influence the risk and possibly the disease progression in individuals susceptible to Type 1 Diabetes Mellitus.

Chapter 2: Rationale

Early diet has been demonstrated to be an influential component in the susceptibility to overt Type 1 Diabetes Mellitus. The mechanisms by which diet is able to alter the progression of diabetes are not fully understood. However, the timing and nature of early dietary exposure in both rodents and humans has been indicated as a critical factor.

To further explore the impact of diet modulation and its potential mechanisms, two immune-modulating diets, a semi-purified casein diet (diabetes protective; Storlein et al. 1996) and a standard chow (NIH-07, diabetes inducing; Scott et al. 1985, Issa-Chergui et al. 1988, Hoorfar et al. 1992) were selected, to study in the BB rat. The BB rat represents an animal model where disease incidence have previously been demonstrated to be altered by early diet intervention.

Along with developing spontaneous diabetes, the BBdp rat is characterized by a number of immunological abnormalities including lymphopenia (Elder et al. 1983, Yale et al. 1985) and altered lymphocyte metabolic activity (Field et al. 1994). Introducing a diabetes-protective, casein-based diet in place of chow is able to influence the immune function of BBdp rats at weaning (Field 1995).

Dietary modulation by semi-purified and chow diets has been indicated to alter varying aspects of intestinal structure and function in BB rats. Reimer et al. (1998) demonstrated that chow and protective semi-purified diets are able to alter small intestinal proglucagon expression, glucose transport capacity, intestinal weight and intestinal length in BBn and BBdp rats. Highly diabetogenic chow alone has been shown to amplify proglucogan presence and glucose transport capacity (SGLT-1, GLUT-2, GLUT-5) in the intestine of BBn rats at 30 days of age (Reimer et al. 1997). Thus, based on the previous work in this field, there is a suggestion that there is a relationship of both diabetes and diet with the immune system and the gastrointestinal tract.

Diet has the capacity to alter intestinal immune function both in BB rats and humans. Specific dietary fats have the ability to alter the presence of suppressive (Th2) cytokine mRNA at the level of the gut and pancreas in the BB rat (Kleemann et al. 1998). Purified casein and soy diets have also been shown to alter peripheral lympohcytes in

young BB rats, in comparison to chow feeding (Field et al. submitted (a)). The fermentable fiber content of the diet can alter T-cell function in GALT (Field et al. submitted (b)). Other factors, including states of vitamin deficiency and malnutrition have been shown to alter GALT function (Langkamp-Henken et al. 1992).

Secretory IgA is a humoral factor with a role in early intestinal development, both in its presence from maternal and endogenous sources. This represents a logical parameter by which diet modulates immune function in the BB rat. IgA acts to limit systemic antigen exposure (Childers et al. 1989), which is critical in both defense and tolerance. IL-2R is a receptor for a cytokine important in the promotion of IgA production (Brandtzaeg 1996) and lymphocyte activation (Takeshita et al. 1992) provides a second potential immune factor that could alter early development of the immune system and the response to diet.

At present, there are no studies demonstrating that diet can alter GALT in the young BB rat, prior to the development of clinical diabetes. Antigen exposure through diet, in the GI tract may have the capacity to modulate the gastrointestinal tract and its immune tissues, as in other tissues (Scott et al. 1997), ultimately impacting systemic immunity and tolerance. As well, the proximity and hormonal links between the pancreas and the gut, the gut and autoimmunity remain to be examined.

Overall Objective and Hypotheses

To determine the influences of diet and genetic susceptibility to Type 1 diabetes upon the structure and immune system of the intestinal tract in a neonatal model.

Through the study of intestinal parameters in neonatal BB rats, it is anticipated that the response to diet will be affected by the BB genotype.

1. Small intestinal structure will be influenced by feeding diets known to alter the incidence of diabetes in the BB rat.

   It is hypothesized that chow feeding will be associated a hypertrophied mucosal structure and that these differences will be greater in the BBdp rat at both

15 and 30 days of age.

2. Small intestinal measures of immunological status will be influenced by feeding diets known to alter the incidence of diabetes in the BB rat.

   It is hypothesized that the relative proportions of IgA bearing immunocytes will increase with chow feeding and will be greater in BBn rats. It is hypothesized that the relative proportions of IL-2R bearing cells will increase with chow feeding and will be greater in BBn rats.

Chapter 3: Materials and Methods

Animals

BioBreeding diabetes prone (BBdp) and non-diabetes prone (BBn) rats were obtained from the Department of Agricultural, Food and Nutritional Science breeding colony (original breeding stock from the Animal Resources Division, Health Protection Branch, Health Canada, Ottawa, Ontario, Canada). Study protocols followed guidelines set by the Canadian Council on Animal Care; all protocol procedures were reviewed and approved by the Faculty of Agriculture, Forestry and Home Economics Animal Policy and Welfare Committee. (Protocol numbers: 97-52B, 97-08B). Animals were group-housed in plastic shoebox cages, exposed to 12 hour light/dark cycle in a temperature and humidity-regulated room. Animals were weaned to the same diet as their respective dams at 21 days of age. For intestinal sampling, animals were selected on the basis of age and BB strain. Both BBn and BBdp rat pups were killed at 15 days (prior to weaning) and 30 days (after weaning) of age to obtain intestinal samples. Briefly, animals were anesthetized with halothane (3-5% at 1.5-2mL O_2/min), a blood sample was obtained via cardiac puncture, and animals were then terminated via cervical dislocation.

Diets

Two diets were utilized in this experiment; a semi-purified casein diet and a standard laboratory chow (NIH-07 Rodent Diet, *Zeigler Brothers, Inc.*, Gardners, PA, USA). The semi-purified diet (similar to the AIN-76 diet) was made in our laboratory, and fed in a powdered form. One of the two nutritionally complete diets was fed to each BBn and BBdp dams, 7-10 days prior to parturition. All animals were provided diet and water (*ad libitum).* At 21 days of age, pups were weaned to the diet of their respective dams. The diet composition is show in Table 1.

Table 1: *Diet Composition*

Diet	*NIH-07*[1,2,3,5,6]	*Semi-purified Casein*[4,6]
Component	*%(w/w)*	*%(w/w)*
Moisture	11.0	5.9
Ash	6.4	4.9
Protein	23.0	26.5
Fat (as triglyceride)	5.5	14.5
(% fatty acids)		
% Saturated Fat	1.3	3.6
% Monounsaturated Fat	1.2	5.0
% Polyunsaturated Fat	2.8	1.6
Carbohydrate (by difference)	54.1	48.2
Sucrose	1.7	<1.0
Dietary Fiber	13.7	11.6
% Insoluble	>99	90
% Soluble	<1	10
Starch (by difference)	38.7	35.6
Total Energy Content (kcal/g)	**3.58**	**4.29**

[1]Macronutrient content supplied as (g/100g): dried skim milk (5), fish meal (10), soybean meal (12), alfalfa meal (4), corn gluten meal (3), ground yellow shelled corn (24.5), ground hard winter wheat (23), wheat middlings (10), Brewer's dried yeast (2), dried molasses (1.5), soybean oil (2.5).

[2]Supplemented with (g/100g): dicalcium phosphate (1.25), ground limestone (0.5), premixes (0.25), sodium chloride (0.5).

[3] Carbohydrate content calculated as amount remaining after subtracting for protein, fat, fiber, ash and moisture (based on October 1997 milling information).

[4] Supplemented (g/kg diet): AIN mineral mix (35), AIN vitamin mix (10), DL-methionine (3) and choline bitartrate (2). Mineral mix content (mg/kg mix): $CaPO_4$ (500), NaCl (74), $K_3C_6H_5O_7 \bullet H_2O$ (220), K_2SO_4 (52), MgO (24), $MnCO_3$ (3.5), $C_6H_5O_7Fe \bullet 3H_2O$ (6), zinc carbonate (1.6), cupric carbonate (0.3), KI (0.01), Na_2SeO_3 (0.01), $CrK(SO_4)_2 \bullet 12H_2O$ (0.55), sucrose (118). Vitamin mix content (mg/kg mix): thiamin hydrochloride (0.6), riboflavin (0.6), pyridoxine hydrochloride (0.7), nicotinic acid (3), D-calcium pantothenate (1.6), folic acid (0.2), D-biotin (0.02), vitamin B_{12} (0.02), D,L-alpha-tocopherol acetate (20), cholecalciferol (0.25), sucrase (973), 1.2×10^5 RE (retinylacetate).

[5] Vitamin and Mineral content supplied: vitamin A (10.00 IU/g), vitamin D_3 (4.00 IU/g), α-tocopherol (35.00 IU/kg), thiamine (14.00 ppm), riboflavin (7.00 ppm), niacin (80.00 ppm), pantothenic acid (20.00 ppm), choline (2000.00 ppm), pyridoxine (10.00 ppm), folic acid (3.00 ppm), biotin (0.15 ppm), vitamin B_{12} (30.00 μg/kg), vitamin K (3.00 ppm), calcium (1.20%), phosphorus (0.95%), potassium (0.80%), sodium (0.33%), magnesium (0.15%), iron (250.00 ppm), zinc (45.00 ppm), manganese (80.00 ppm), copper (10.00 ppm), cobalt (0.70 ppm), iodine (1.80 ppm).

[6] Dietary Analysis: Analysis was performed using the following AOAC methods; moisture (14th edition, 1989, sec. 14.003, p.249), ash (15th edition, 1990, method 923.03), total dietary fiber, insolube/soluble fiber (16th edition, 1995, method 991.43), total fat (NLEA) and % fatty acids (16th edition, 1995, method 991.43), protein (AOAC Kjeldhal method, using N x 6.25).

Intestinal Sampling

All samples were collected between 12:00 and 16:00 hours. The small intestine

from the ligament of Treitz to the ileocecal valve was rapidly excised from the length of the intestine. Length determinations were made upon this intact segment, under the tension of a known weight (5g) to ensure even extension for rats older than 25 days of age. Length determinations of the intact intestinal segment for rats less than 25 days of age were taken without the addition of weight. The entire intestinal segment was then transferred to an iced surface, divided into three approximately equal sections; the proximal jejunum, a middle segment and the terminal ileum. These three sections were then flushed with cold (4°C) phosphate buffered saline (pH=7.4), blotted and individually weighed. The weighed segments were then further subdivided into samples for histology; 3-5cm of the most proximal jejunum and terminal ileum were collected into histological cassettes (*Fisher*, Pittsburgh, Pennsylvania, USA) and placed 10% w/v phosphate-buffered formalin (*Fisher*, Nepean, Ontario, Canada). A small segment was also retained for dry weight determination. Each segment retained for dry weight determination was weighed, placed tin foil (of known weight) and dried overnight at 60°C. The remaining segments were placed in 7ml plastic scintillation vials, snap frozen in liquid nitrogen, and stored in a -70°C freezer.

Histological Tissue Preparation

All small intestinal samples collected for histological evaluation were maintained in 10% w/v phosphate-buffered formalin (*Fisher*, Nepean, Ontario, Canada) for a period of 72-96 hours. Following fixation, the tissues were washed under running tap water, overnight. Upon completion of washing, tissues were dehydrated and cleared, as previously described (Humanson 1979). Briefly, tissues were dehydrated through a serious of graded ethanol and two cycles of clearing through xylene:

Table 2: *Tissue Processing Solution Progression*

Order	*Solution*	*Time*
1	50%(v/v) Ethanol	1 hour
2	70%(v/v) Ethanol	1 hour
3	95%(v/v) Ethanol	1 hour
4	Absolute Ethanol	30 minutes
5	Absolute Ethanol	30 minutes
6	Xylene	30 minutes
7	Xylene	30 minutes

Following the preparation process, tissues were immersed in melted paraffin for a period of 30 minutes to 2 hours, to enable the adherence of paraffin to the tissue sample. Samples were then embedded in paraffin (*Oxford Labware*, St. Louis, Missouri, USA), in tissue molds which had been preheated. Paraffin was supported with embedding rings (*Simport*, Saint-Mathiew-de-Beloeil, Québec, Canada). The embedded samples were then rapidly cooled, and removed from the embedding mold.

Sectioning

Embedded tissues were serially into 4-6 μm sections. Sections were then placed upon poly-L-lysine coated (*Sigma*, St. Louis, MO, USA) glass slides (*Fisher*, Pittsburgh, PA).

Staining

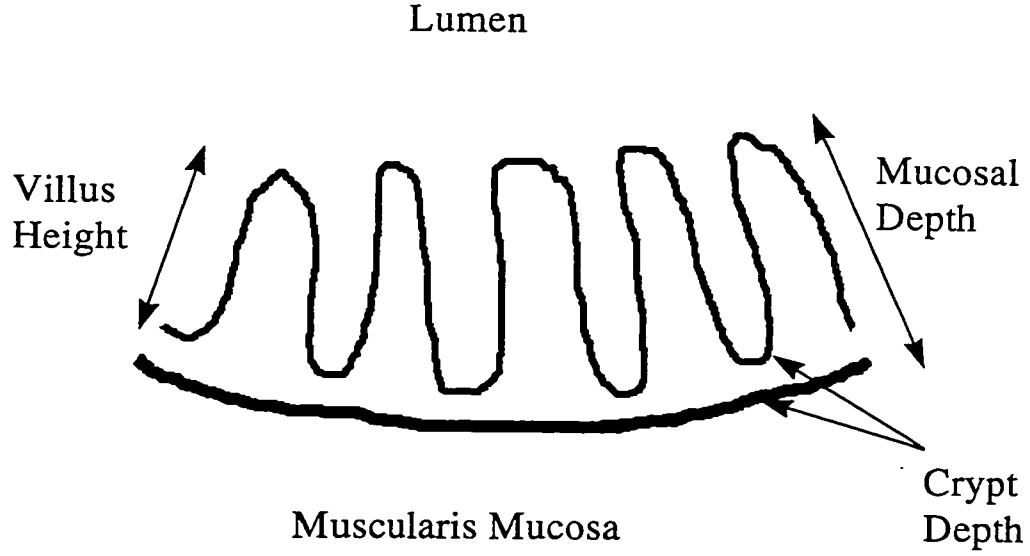
Tissue sections were deparaffinized and rehydrated through xylene and graded ethanols. Briefly, the process included xylene (*Fisher*, Nepean, Ontario, Canada) (3 minutes), absolute ethanol (2 minutes), 95% (v/v) ethanol (2 minutes), 70% (v/v) (2 minutes), and running water (3-5 minutes). Routine hematoxylin and eosin staining was completed using regressive Harris Hematoxylin, and counterstaining with eosin. The staining procedure included the following sequential steps: hematoxylin staining (90 seconds), a 3 minute wash in running tap water; differentiation in acid alcohol (20

seconds), a 2-3 minute wash in running tap water, 1-2 minutes of bluing with Scott's Tap water (please see Appendix 1), a 3-5 minute wash and a 1 minute counterstain with eosin.

Evaluation

Under a light microscope (Leitz Dialux 20, Wetzlar, Germany) at 100X (10X objective, 10X eye piece) magnification, small intestinal mucosal depth, villus height and crypt depth were determined with Northern Exposure software (*Epix Imaging Inc.*, Mississauga, Ontario, Canada), and recorded in Microsoft Excel. Each sample slide had been coded and examined in random order. Ten randomly selected, intact villi were evaluated for mucosal measures. Mucosa overlying Peyer's patches or lymphoid nodules was not included in the evaluation. The measure of mucosal depth was defined as the distance between villus tip and the muscularis mucosa (Wheater 1987, please see Figure 1). Villus height was determined by measuring from the villus tip to a point perpendicular between adjoining crypts. Crypt depth was determined by the difference between mucosal depth and villus height measures.

Figure 1. Schematic of mucosal measures for crypt depth, villus height and mucosal depth.



Immunostaining

Sectioned samples on slides were obtained, and deparaffinized/rehydrated as previously described in histological staining. Tissue samples were subjected to an eight minute, 50-percent power unmasking procedure of microwave treatment (650W

microwave, *Toshiba*, Tadehara, Fuji-shi, Shiuoka-ken, Japan) in a citrate buffer (pH 6.0), followed by a five minute wash in PBS. Endogenous peroxidase activity was blocked with a 30-minute incubation in 0.03%(v/v) hydrogen peroxide (*Fisher*, Fairlawn, New Jersey, USA)/methanol. Non-specific binding was blocked with the addition of horse serum (a twenty-minute incubation). Primary antibodies were applied for a thirty minute period; mouse anti-rat monoclonal antibodies were applied for both IgA (*Sigma*, St. Louis, Missouri, USA) and IL-2R (*Cedarlane*, Hornby, Ontario, Canada). Preliminary experiments utilizing anti-TCR antibodies utilized mouse anti-rat αβ TCR and mouse anti-rat γδ TCR (*Pharmingen*, Mississauga, Ontario, Canada). One control slide per animal was randomly designated, and was processed in same manner as all those subject to IgA or IL-2R staining, with the following modifications: a primary antibody was not applied, and wash periods were conducted separately from other slides to prevent contamination. A separate isotype control was prepared utilizing only mouse-prepared anti-IgG1 antibodies. Mouse anti-sheep CD8 (*VMRD Inc.*, Pullman, Washington, USA) and mouse anti-canine CD40 (*Serotec*, Mississauga, Ontario, Canada).

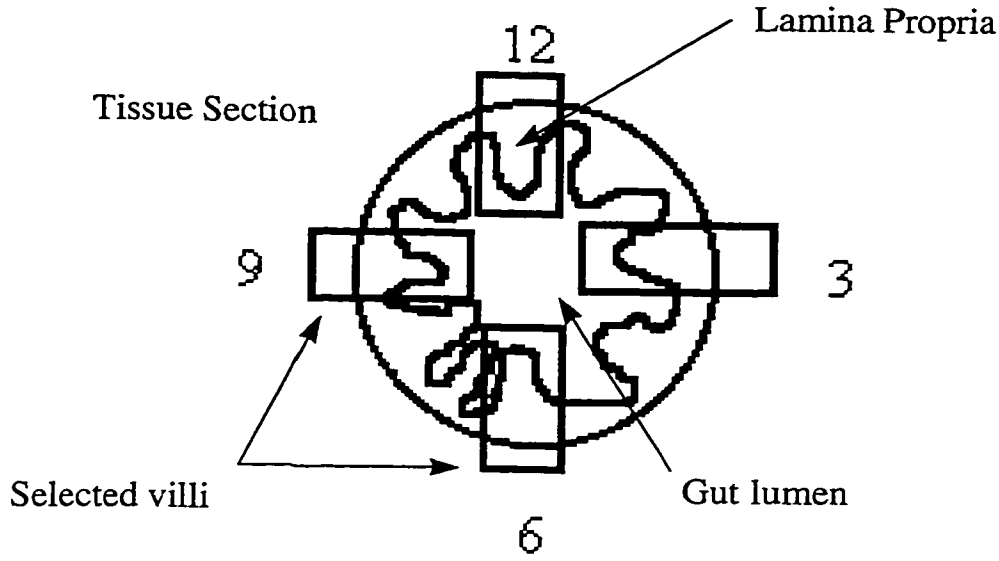
All specified immunological reagents were made up in PBS; wash periods were also conducted in PBS. The secondary antibody (horse anti-mouse IgG), blocking serum and peroxidase reagents were assembled in an immunohistochemistry kit (*Vector Laboratories*, Burlington, Ontario, Canada). A peroxidase-activated colorimetric reagent was applied, and generated a blue-black stain (Vector SG, *Vector Laboratories*, Burlington, Ontario, Canada). All incubation periods were carried out in a humidified chamber. Once immunostained, samples were counterstained with eosin, dehydrated, cleared in xylene and mounted with D.P.X. Mountant (*B.D.H. Laboratory Chemicals Division*, Poole, England).

Immunological Evaluation

All immunological samples were examined in a blinded and randomized manner, by the same individual. Individual sections were examined using a standardized-orientation manner, such that four intact villi were examined per section (in four standardized positions, occupying positions found on a universal 12-hour timepiece, please see Figure 2). Three tissue sections were examined per slide. Only positively

stained cells within the lamina propria region of the intestinal sections were quantified. The examination of sections was completed using a Leitz Dialux (Wetzlar, Germany) microscope at 400X magnification.

Figure 2. Immunohistochemical quantification procedure schematic.



Statistical Analysis

All statistical analysis of morphological and immunological data was completed using the SAS (Statistical Analysis System) statistical software (Version 6.11, *SAS Institute Inc.*, Cary, NC, USA). Means are expressed ± SEM, and the level of significance was set at $p \leq 0.05$. Values were analyzed for the effects of diet and BB strain using a 2-way ANOVA. Differences were identified using least square means/pdiff. Data was also examined with respect to sex and age (where appropriate) using a 3-way ANOVA; data that did not differ significantly ($p > 0.05$) by sex was combined. Although differences between sex in other morphological and immune parameters in neonatal BB rats has not previously been reported, the absence of sex differences was confirmed by the aforementioned 3-way ANOVA. Immunological data with respect to individual sexes in the results chapter is presented in support of this procedure.

Chapter 4: Results

Animal Characteristics

Day 15 (Table 3)

As indicated in Table 3, at 15 days of age, body weight differed only between diet groups. Chow fed BBn (26.9±0.9g) and BBdp (27.7±1.1g) rats demonstrated greater body weight than their casein fed BBn (21.5±2.0) and BBdp (21.5±0.8) counterparts ($p \leq 0.0001$). No strain difference was found in body weight within same diet groups. Intestinal wet weight at 15 days of age varied between diet groups; both BBn and BBdp rats displayed greater intestinal wet weights when chow fed ($p \leq 0.001$). Adjusted for body weight, intestinal wet weight did not differ between diet or strain groups.

Measures of intestinal dry weight followed a similar pattern; both estimated jejunoileal dry weight and adjusted dry weight per body weight differed between chow and casein fed animals of the same strain at $p \leq 0.0001$. The average dry intestinal weight differed only between BBn rats; chow fed BBn rats had an average 22.0±0.3% dry weight and casein fed BBn rats had an average of 20.2±0.4% ($p \leq 0.05$).

Intestinal length and intestinal length per body weight unit varied among treatment groups in the following fashion. Chow fed animals had greater jejunoileal length in comparison to casein fed ($p \leq 0.0004$) and greater jejunoileal length adjusted for body weight ($p \leq 0.0009$). BBn rats had longer intestines than BBdp animals in both diet groups both when expressed as jejunoileal length and when length was adjusted per bodyweight unit (strain effects of $p \leq 0.001$ and $p \leq 0.007$, respectively). Measures of both wet and dry intestinal weight expressed per cm of intestinal length were greater with chow feeding, in comparison to casein feeding, regardless of strain ($p \leq 0.0001$, wet weight; $p \leq 0.0001$, dry weight).

Table 3. Animal and intestinal characteristics at 15 days of age.

Diet	*Chow*		*Casein*	
Strain	**BBn** (n=8)	**BBdp** (n=6)	**BBn** (n=6)	**BBdp** (n=6)
Body Weight (g)	26.9±0.9[a]	27.1±1.1[a]	21.5±1.1[b]	21.5±0.8[b]
Wet Weight (g)	0.82±0.05[a]	0.83±0.03[a]	0.58±0.05[b]	0.55±0.03[b]
Wet Weight/Body Weight (g/g)	0.031±0.001	0.030±0.002	0.030±0.001	0.028±0.001
Est. Dry Weight (g)	0.18±0.010[a]	0.18±0.011[a]	0.12±0.013[b]	0.12±0.004[b]
Est. Dry Weight/Body Weight ($x10^{-3}$ g/g)	6.62±1.2[a]	6.39±0.7[a]	5.33±0.5[b]	5.51±0.6[b]
Average % Dry Weight	22.0±0.3[a]	21.3±0.6[ab]	20.2±0.4[b]	21.3±0.4[ab]
Length (cm)	44.2±1.1[a]	40.8±1.1[b]	40.5±1.3[b]	36.5±0.7[c]
Length/Body Weight (cm/g)	1.66±0.04[bc]	1.50±0.06[c]	1.95±0.12[a]	1.71±0.04[b]
Wet Weight/Length ($x10^{-2}$ g/cm)	1.83±0.8[a]	2.03±1.0[a]	1.42±0.9[b]	1.52±0.6[b]
Dry Weight/Length ($x10^{-3}$ g/cm)	4.0±1.4[a]	4.3±1.0[a]	2.8±0.2[b]	3.2±0.1[b]

Unlike superscripts represent significant difference ($p \leq 0.05$) within a diet-strain group, as calculated by lsmeans pdiff.

Values presented are those of whole animal and intestinal measures, means ± SEM. Values for sample size (n) refer to the mean of each sex within each litter; chow fed BBn (n=8), chow fed BBdp (n=6), casein fed BBn (n=6), and casein fed DP (n=6). Measures of wet weight were taken directly; measures of estimated dry weight were calculated from the average dry weight content of three sample sections of standard

regions of the intestine measured both wet and dry, following a 24 hour drying period. The calculated dry matter content was then applied to the entire intestinal weight, region by region.

Day 30 (Table 4)

At 30 days of age, chow fed BBn (86.8±4.1g) and BBdp (83.2±7.2g) rats had higher body weights than casein fed BBn (60.5±4.5g) rats ($p \leq 0.01$). Casein fed BBdp rats had body had a mean body weight that differed only from casein fed BBdp rats, (79.8±4.3g). Intestinal wet weight at 30 days of age varied between diet groups ($p \leq 0.0001$) in both BBn and BBdp rats, with chow fed animals displaying greater intestinal wet weight than those fed casein. Chow fed animals did also demonstrate a strain difference in intestinal wet weight; BBn jejunoileum weight (4.07±0.14g) being significantly greater ($p \leq 0.02$) than BBdp jejunoileal weights (3.55±0.2). Casein fed animals displayed greater jejunoileal weight for BBdp rats compared to BBn rats. Adjusted for body weight, however intestinal wet weight differed only between chow fed BBn rats and casein fed BBdp rats.

Estimated dry weight measures differed by diet in both BBn and BBdp rats ($p \leq 0.0001$). Further, both chow and casein fed rats exhibited a differential strain effect; with greater intestinal dry weight for chow fed BBn rats in comparison to BBdp rats, and greater intestinal dry weight for casein fed BBdp rats versus BBn rats. As dry weight measures were adjusted for body weight, the differences between diet groups were eliminated, and dry weight per body weight differed only between chow fed BBn and casein fed BBdp rats ($p \leq 0.05$). The average percent dry weight for casein fed BBdp rats was greater than all other diet strain groups ($p \leq 0.05$).

Intestinal length (measured under tension) and intestinal length per body weight unit differed in the following manner. Chow fed rats had the longest ($p \leq 0.05$) intestine, compared to the intestinal length of the other three groups. However, casein fed BBn rats displayed the greatest adjusted length per body weight. Diet did not affect intestinal length measures in BBdp rats. Chow feeding resulted in greater intestinal length in BBn rats, above that seen in BBdps. There was no difference in intestinal length between BBn and BBdp casein fed rats. However, adjusted for body weight, intestinal length did differ

between casein fed BBn and BBdp rats, with greater measures in BBn rats. Measures of wet intestinal weight expressed in relation to intestinal length differed by diet ($p \leq 0.0001$) in BBn rats, but did not in BBdp rats. Intestinal wet weight/intestinal length did not differ among BBn and BBdp rats within a diet group. Intestinal dry weight/intestinal length differed by diet in BBn rats ($p \leq 0.0001$), but did not differ in BBdp rats. This measure differed among strains within the casein diet group ($p \leq 0.02$), but did not among chow fed rats.

Age affected all intestinal and body weight parameters measured ($p \leq 0.05$, statistics not illustrated). The only parameters not following this pattern were those of percent dry weight, which did not have diet-strain differences between age categories. Those groups not exhibiting differences between 15 and 30 days of age were casein fed BBn and BBdp rats, and chow fed BBdp rats, in the parameter 'average percent dry weight of the intestine.

Table 4. Animal and intestinal characteristics at 30 days of age.

Diet	*Chow*		*Casein*	
Strain	**BBn** (n=14)	**BBdp** (n=8)	**BBn** (n=6)	**BBdp** (n=6)
Body Weight (g)	86.8±4.1[a]	83.2±7.2[a]	60.5±4.5[b]	79.8±4.3[a]
Wet Weight (g)	4.07±0.14[a]	3.55±0.18[b]	2.76±0.13[c]	3.19±0.20[bc]
Wet Weight/Body Weight (g/g)	0.048±0.001[a]	0.045±0.002[ab]	0.047±0.002[ab]	0.042±0.001[b]
Est. Dry Weight (g)	0.83±0.03[a]	0.73±0.05[b]	0.56±0.02[c]	0.68±0.04[b]
Est. Dry Weight/Body Weight ($x10^{-3}$ g/g)	9.62±0.2[a]	8.88±0.3[ab]	9.47±0.5[ab]	8.64±0.2[b]
Average % Dry Weight	20.3±0.1[a]	20.±0.3[a*]	20.2±0.4[a*]	21.2±0.2[b*]
Length (cm)	86.9±1.7[a]	82.6±1.3[b]	78.3±1.8[b]	79.6±0.6[b]
Length/Body Weight (cm/g)	1.02±0.03[a]	1.04±0.08[a]	1.33±0.08[b]	1.02±0.05[a]
Wet Weight/Length ($x10^{-2}$ g/cm)	4.68±1.0[a]	4.29±1.9[ab]	3.52±1.5[c]	3.98±2.2[bc]
Dry Weight/Length ($x10^{-3}$ g/cm)	9.5±0.2[a]	8.8±0.5[ab]	7.1±0.3[c]	8.4±0.4[b]

Unlike superscripts represent significant difference ($p \leq 0.05$) within a diet-strain group, as calculated by lsmeans pdiff. * Superscripts indicate the absence of difference between age groups.

Values presented are those of whole animal and intestinal measures, means ± SEM. Values for sample size (n) refer to the mean of each sex within each litter; chow fed BBn (n=14), chow fed BBdp (n=8), casein fed BBn (n=6), and casein fed DP (n=6). Measures of wet weight were taken directly; measures of dry weight were calculated from the average dry weight content of three sample sections of standard regions of the

intestine measured both and wet and following a 24 hour drying period. The calculated dry matter content was then applied to the entire intestinal weight, region by region.

Morphological Measures

Day 15

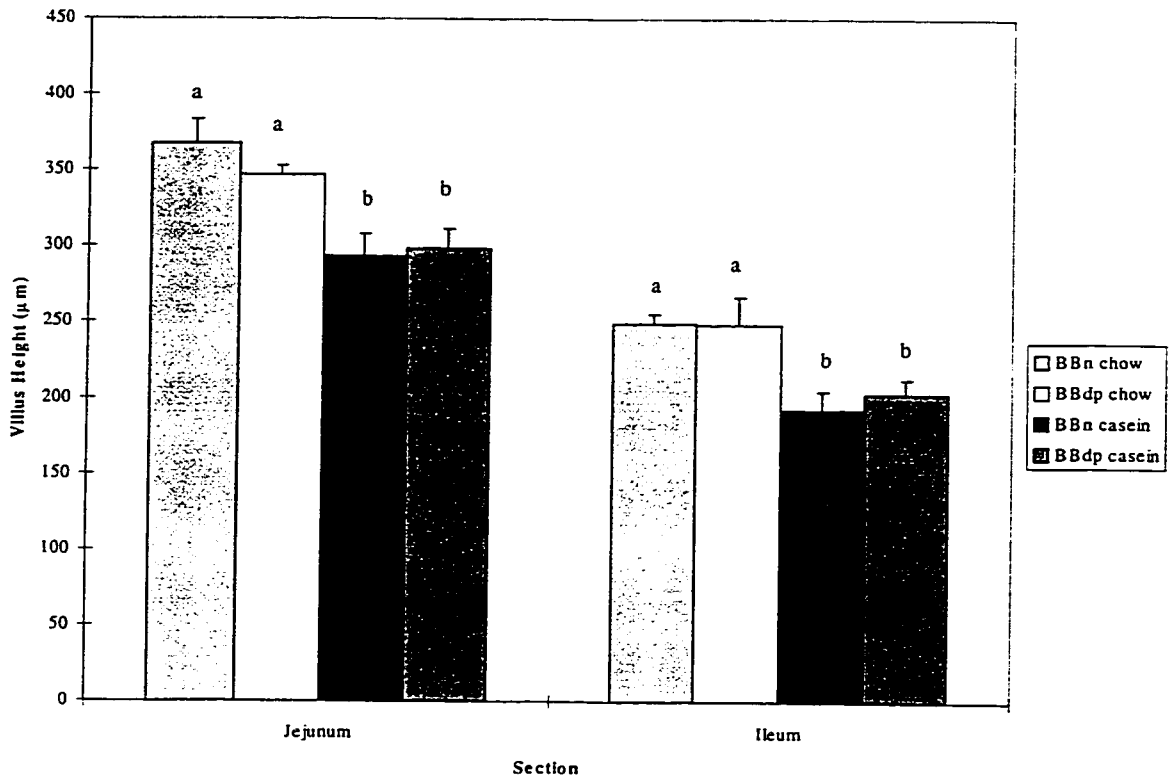
Table 5. Mucosal measures of 15 day old BBn and BBdp rats fed chow or casein.

Diet	*Chow*		*Casein*	
Strain	**BBn**	**BBdp**	**BBn**	**BBdp**
Jejunal Mucosal Depth (μm)	440 ± 18^{a}	426 ± 7^{a}	361 ± 16^{b}	377 ± 13^{b}
Jejunal Crypt Depth (μm)	72 ± 3^{ab}	80 ± 4^{a}	67 ± 3^{b}	78 ± 2^{a}
Ileal Mucosal Depth (μm)	318 ± 7^{a}	326 ± 21^{a}	256 ± 15^{b}	270 ± 13^{b}
Ileal Crypt Depth (μm)	68 ± 3^{ab}	77 ± 4^{a}	63 ± 4^{b}	67 ± 4^{b}

Values that do not share the same superscripts within an intestinal section and mucosal measure are significantly different, $p\leq0.05$. Values presented are those of means of each diet strain group (n=12 animals), ± SEM.

Histology of small intestinal mucosa was evaluated for three characteristics, total mucosal depth, villus height and crypt depth. At 15 days of age, chow fed animals demonstrated greater mucosal depth than casein fed animals in both the BBn and BBdp strain in both the jejunum and ileum (Table 5). Chow fed BBn (367±16μm) and BBdp (347±7μm) rats displayed greater villus height than their casein fed BBn (294±15μm) and BBdp (299±13μm) counterparts in the jejunum ($p\leq0.0001$) (Figure 3). Diet differences were present at both the jejunum and ileum in both diet groups in villus height (Figure 3). Jejunal crypt depth was significantly less in casein fed BBn versus all other groups except chow fed BBn rats (Table 5). Ileal crypt depths were statistically unaffected by diet

(Table 5).



Bars that do not share like superscripts for jejunum or ileum are significantly different, $p \leq 0.05$.

Figure 3. Measures of villus height in the jejunum and ileum of 15 day old BBn and BBdp rats, maintained with dams fed casein and chow. Values are presented as means ± SEM (n=12 for each diet/strain group within an intestinal region).

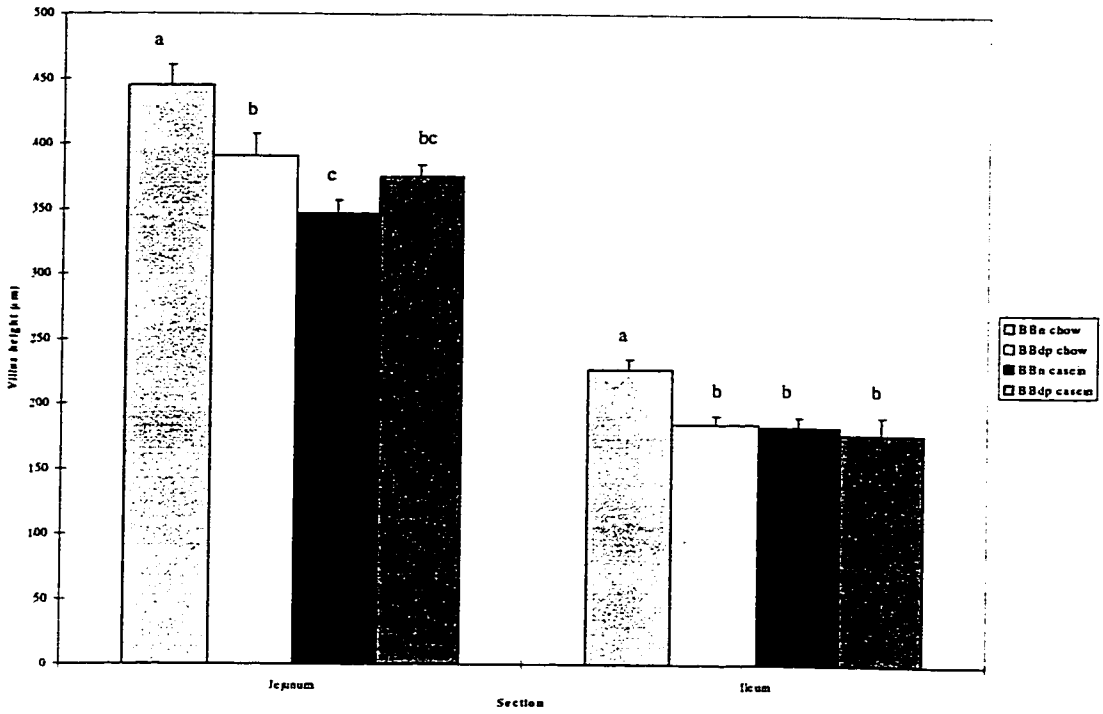
Day 30

Table 6. Mucosal measures of 30 day old BBn and BBdp rats fed chow or casein.

Diet	*Chow*		*Casein*	
Strain	**BBn**	**BBdp**	**BBn**	**BBdp**
Jejunal Mucosal Depth (μm)	632±20[a]	539±25[b]	501±12[b]	536±9[b]
Jejunal Crypt Depth (μm)	187±7[a]	148±12[b]	155±4[b]	161±20[b]
Ileal Mucosal Depth (μm)	368±14[a]	315±13[b]	312±11[b]	300±15[b]
Ileal Crypt Depth (μm)	141±6[a]	130±9[ab]	129±5[ab]	122±5[b]

Values that do not share the same superscripts within an intestinal section and mucosal measure are significantly different, $p \leq 0.05$. Values presented are those of means of each diet strain group (n=12 animals), ± SEM.

All measured mucosal parameters were greater in 30 day old rats, compared to 15 day old rats ($p \leq 0.002$). At 30 days of age, the greatest jejunal mucosal depth was seen in chow fed BBn rats (Table 6). This measure was significantly greater than all other groups. The diet difference in this is measure in BBdp rats, seen at 15 days of age was no longer present. The greatest villus height was displayed in BBn, chow fed rats (Figure 4). Compared to casein fed, BBn animals fed chow had a higher villus height (445±16μm vs. 346±10μm) (Figure 4). There was no diet effect in BBdp rats (Figure 4). The deepest crypts were found in the jejunum of chow fed BBn rats. This measure in BBn rats was significantly deeper than all other groups (Table 6). For the ileum, there was no diet or strain effect fed BBn rats in crypt depth.



Bars that do not share like superscripts for jejunum or ileum are significantly different, $p \leq 0.05$.

Figure 4. Measures of villus height in the jejunum and ileum of 30 day old BBn and BBdp rats, fed casein and chow. Values are presented as means ± SEM (n=12 for each diet/strain group within an intestinal region).

Immunological Measures

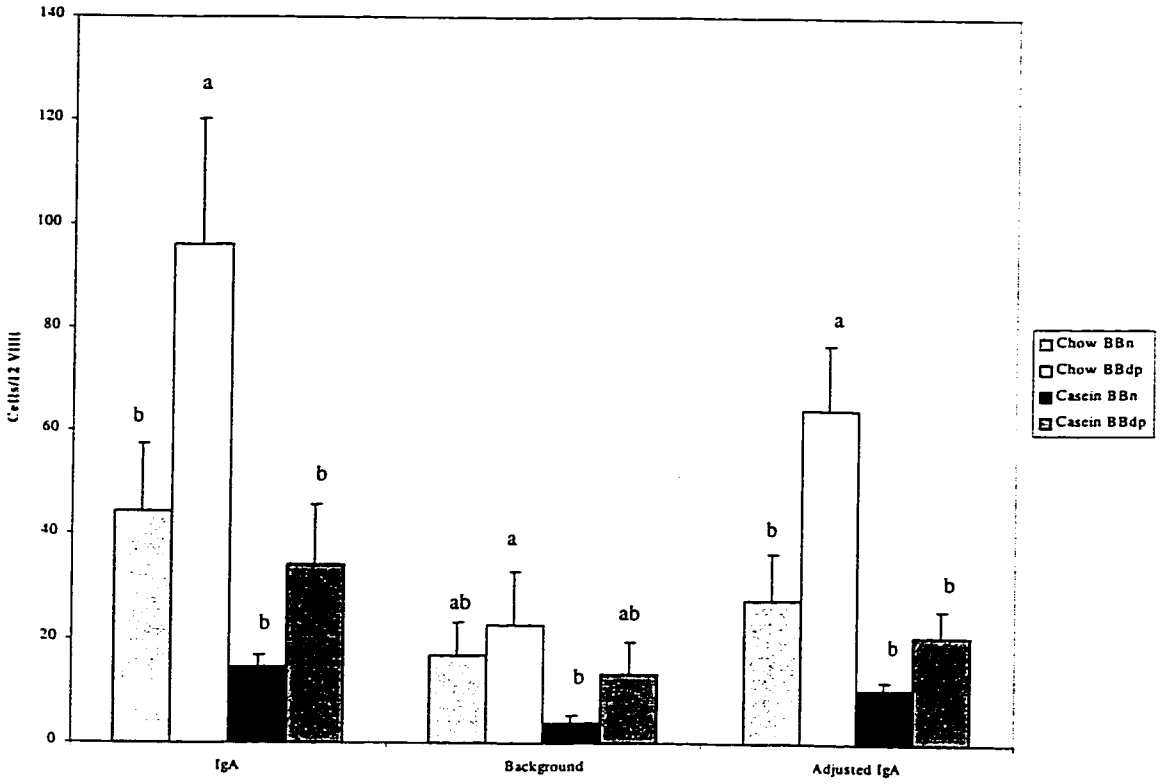
Day15 (Please see Appendix 3)

Testing for the presence of several immunological markers was evaluated in 15 day old BBn and BBdp rats. No detectable levels of the following markers were found in 15 day old animals: TCR αβ, TCR γδ, and IL-2R. A limited presence of IgA bearing regions were detected in a few animals, and but was not different by strain or diet.

Day 30 (Please see Appendix 3)

Unlike animals at 15 days of age, animals at 30 days of age displayed distinctively stained IgA and IL-2R bearing cells within the lamina propria.

IgA



Unlike superscripts represent values of significant difference, p≤0.05.

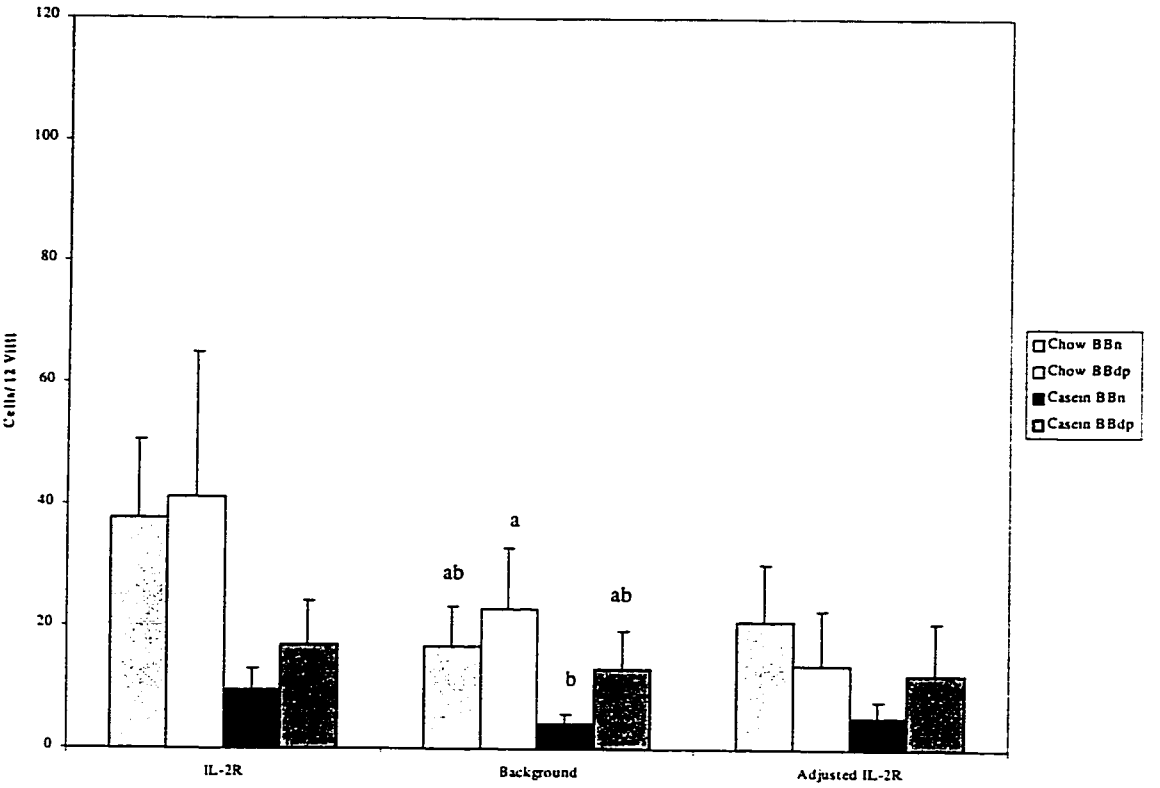
Figure 5. The prevalence of IgA bearing lamina propria immunocytes within the jejunum of 30 day old BBn and BBdp rats fed chow or casein. Values represent the sum of IgA-bearing immunocytes within the lamina propria regions of 12 villi. IgA represents the raw or unadjusted cell count; adjusted IgA represents the subtraction of background values from raw cell counts. Values are presented as means ± SEM (n=12 per diet/strain group for BBn rats and n=11 per diet/strain group for BBdp rats).

At 30 days of age, at the jejunum, lamina propria IgA data that did not display sex differences, therefore the male and female rats have been combined. Individual data for each sex is presented in Tables 7 and 8. The highest number of cells expressing IgA was observed in chow fed, BBdp rats, (Figure 5) and this differed from BBdp casein and both BBn diet groups. This difference in chow fed BBdp rats also included the greatest expression of raw (unadjusted) IgA bearing cell counts, and adjusted (for background) cell counts (Figure 5). The number of IgA bearing cells in the jejunum of casein fed BBdp rats did not differ from casein-fed BBn rats.

Table 7. Comparison of IgA bearing cells by sex. Values presented are those of cells/ 12 villi, ± SEM; unlike superscripts represent significant differences within a sex group ($p \leq 0.05$) determined by a two-way ANOVA of diet and strain. Three-way ANOVA comparison of sex did not differ significantly.

Sex	Strain	Diet	IgA (cells/12 villi±SEM)	Background (cells/12 villi±SEM)	Adjusted IgA (cells/ 12 villi±SEM)	Sample size
M	BBn	Chow	49.3±21.9^{ab}	15.0±5.8	34.3±17.6^{ab}	n=6
M	BBdp	Chow	85.4±9.4^{a}	29.8±18.6	56.7±9.9^{a}	n=5
M	BBn	Casein	18.8±4.3^{b}	6.8±2.2	12.0±2.2^{b}	n=6
M	BBdp	Casein	41.2±18.2^{ab}	7.8±2.2	16.6±5.0^{ab}	n=6
F	BBn	Chow	39.5±16.4^{ab}	18.7±13.3	20.8±5.6^{b}	n=6
F	BBdp	Chow	105.0±40.9^{a}	15.9±9.2	71.9±24.1^{a}	n=6
F	BBn	Casein	11.5±1.8^{b}	0.2±0.2	10.2±2.1^{b}	n=6
F	BBdp	Casein	42.7±20.4^{ab}	18.5±12.2	24.2±8.7^{b}	n=6

IL-2R



Unlike superscripts represent values of significant difference, $p \leq 0.05$.

Figure 6. The prevalence of IL-2R bearing lamina propria immunocytes within the jejunum of 30 day old BBn and BBdp rats fed chow or casein. Values represent the sum of IL-2R bearing immunocytes within the lamina propria regions of 12 villi. IL-2R represents the raw or unadjusted cell count; adjusted IL-2R represents the subtraction of background values from the raw cell count. Values are presented as means ± SEM (n=11 for each diet/strain group within the following exception: chow BBn, where n=12).

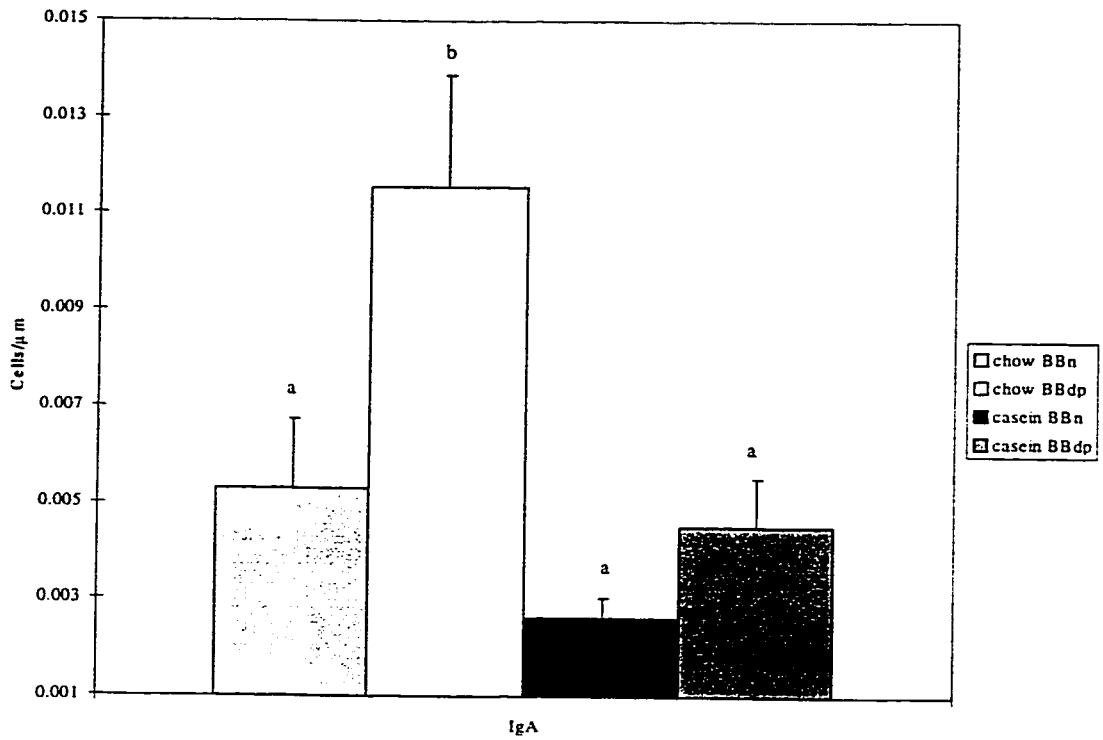
No significant diet or strain effect were seen in the expression of IL-2R, either in raw or adjusted counts (Figure 6). Differences in background counts are examined in Table 8.

Table 8. Comparison of IL-2R bearing cells by sex. Values presented are those of cells/ 12 villi, ± SEM; unlike superscripts represent significant differences within a sex group ($p \leq 0.05$) determined by a two-way ANOVA of diet and strain. Three-way ANOVA comparison of sex did not differ significantly.

Sex	Strain	Diet	IL-2R (cells/12 villi±SEM)	Background (cells/12 villi±SEM)	Adjusted IL-2R (cells/ 12 villi±SEM)	Sample size
M	BBn	Chow	30.0±11.1	15.0±5.8	15.0±8.0	n=6
M	BBdp	Chow	70.3±41.1	29.8±18.6	17.5±14.8	n=6
M	BBn	Casein	12.0±6.2	6.8±2.2	5.2±5.0	n=6
M	BBdp	Casein	10.3±4.1	7.8±2.2	20.1±17.3	n=5
F	BBn	Chow	45.7±24.1	18.7±13.3	27.0±17.2	n=6
F	BBdp	Chow	21.8±15.7	15.9±9.2	13.4±10.3	n=5
F	BBn	Casein	6.8±1.5	0.2±0.2	5.2±0.7	n=5
F	BBdp	Casein	22.8±12.5	18.5±12.2	4.3±0.7	n=6

Jejunal IgA Expression per Villus Height (Figure 7)

Chow fed BBdp rats displayed the greatest average number of IgA bearing cells per micrometer of villus height, significantly higher than BBn rats or casein fed BBdp rats.



Bars with unlike superscripts are significantly different, $p \leq 0.05$.

Figure 7. Examination of IgA bearing lamina propria immunocytes per unit of intestinal villus height within the jejunum of 30 day old BBn and BBdp rats fed chow or casein. Values represent the adjusted average number of IgA bearing immunocytes within the lamina propria region per villus, per unit of villus height. Values are presented as means ± SEM (n=11 per diet/strain group for BBn rats and n=12 per diet/strain group for BBdp rats).

Jejunal IL-2R Expression per Villus Height (Figure 8)

IL-2R expression did not differ between groups.

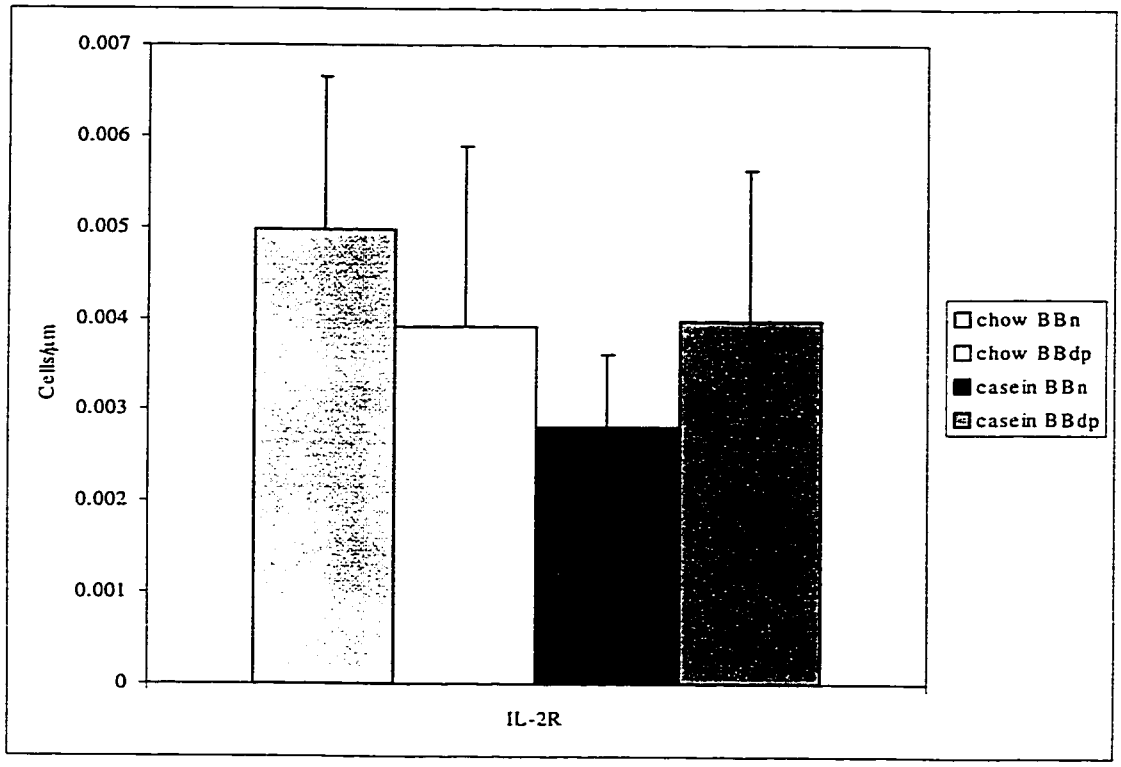


Figure 8. Examination of IL-2R bearing lamina propria immunocytes per unit of intestinal villus height within the jejunum of 30 day old BBn and BBdp rats fed chow or casein. Values represent the adjusted average number of IL-2R bearing immunocytes within the lamina propria region per villus, per unit of villus height. Values are presented as means ± SEM (n=11 for each diet/strain group with the following exceptions; BBdp chow, n=9).

Table 9. Comparison of background immunostaining with the application of two different mouse-harvested (non-rat antigen targeted) antibodies.

Strain	Diet	Average Background (cells/villus) ±SEM	Sample Size
BBn	Chow	0.5±0.1	n=5
BBdp	Chow	1.7±0.5	n=4
BBn	Casein	0.1±0.1	n=6
BBdp	Casein	0.5±0.1	n=3

Antibodies applied were: Mouse Anti-Sheep CD8 (*VMRD Inc.*, Pullman, Washington, USA) and Mouse Anti-Canine CD40 (*Serotec*, Mississauga, Ontario, Canada). Three-way ANOVA statistical analysis indicated antibody target did not alter background measures ($p \leq 0.4$).

The presence of background staining was greatest in chow fed BBdp rats, and was affected by both dietary treatment ($p \leq 0.003$) and BB strain ($p \leq 0.003$).

Chapter 5: Discussion

Animal Characteristics

In general, chow fed animals displayed greater body weight and intestinal weight than their casein fed counterparts at 15 days of age. Strain differences within a diet group were only present in measures of intestinal length. At 30 days of age, the body weight and intestinal length measures of animals were similar to those reported by Reimer et al. 1997 and Reimer et al. 1998. Other studies have reported diet effects upon body weight, such that chow fed rats have greater measures of body weight than those fed semi-purified diets at 30 days of age (Field (a) et al. submitted), but this was not seen in the present study. Overall, measures of intestinal weight at 30 days of age were highest in chow fed BBn rats, and lowest in casein fed BBn rats. The smaller intestinal weights of casein fed BBn rats were normalized with adjustments for body weight.

The diet effects upon intestinal weight and body weight are likely due to several factors, associated with the nutrient content of the diets fed. At 15 days of age, prior to weaning, litter size may ultimately influence the body weight of the litters. Although diet composition should not influence the amount of milk produced by dams (as both diets are nutritionally complete), larger litters may result in smaller milk consumption per pup within a litter. Also, the potential exists for some early consumption of solid diet by pups as they examine their environments. However, only milk content was observed in the stomach in these young animals, at termination. At 30 days of age, diet energy density (Field (a) et al. submitted), fiber content (Field (b) et al. submitted), and immune stimulating properties of several dietary constituents (Alexander 1995) may influence body and intestinal weight. Smaller intestines and lower body weight measures in casein fed animals may involve several factors, including lower dietary immunological stimulation, as immunosuppressant treatment results in smaller intestinal measures (Cummins et al. 1989), and suggest a possible mechanism. The lower energy content of chow on a gram per gram basis (in comparison to casein), and yet higher body and intestinal weight association with chow feeding may be attributed an increased absolute fiber intake. The effects of the higher fermentable content of chow on gut adaptation, and ultimately nutrient uptake may also increase intestine and total body weight (Reimer et al. 1998, Field (a) et al. submitted).

Morphological Data

It was hypothesized that chow feeding would be associated with hypertrophied mucosal structure and that BBdp rats would have larger measures of mucosal structure at both 15 and 30 days of age. As hypothesized, the BB rats responded to casein and chow in different manners. At 15 days of age, there were pronounced increases in mucosal structure size in the mucosa of chow fed versus casein fed rats, however, no strain effects were found on any of the mucosal measures. At 30 days of age, BBn rats fed chow had greater villus height and mucosal depth than casein fed BBn rats and BBdp fed rats. Finally, BBn rats demonstrated intestinal histological changes to diet whereas BBdp rats did not. The hypothesized hypertrophy was not detected in BBdp rats; however, older streptozotocin-diabetic animals have been reported to have larger intestinal structure (Zoubi et al. 1995). As these studies in very young BB rats occur prior to the introduction of diabetes, intestinal hypertrophy may be present at a later time point, closer to disease onset.

The difference in mucosal structure in response to the dietary challenge possessed by the chow diet in BBn rats may indicate an ability to adapt functional characteristics. The chow diet contains greater carbohydrate and fermentable fiber than the casein diet. The casein diet is higher in fat, and lower in fermentable fiber then the chow diet to diet. Dietary macronutrients represent a challenge to the gut. The increased body weight and intestinal weight in chow fed BBn rats suggest an ability of non-diabetes prone rats to respond to the higher dietary carbohydrate load. Consistent with this hypothesis, it was found that the capacity for sugar (glucose, galactose, fructose) transport and proglucagon expression (Reimer et al. 1997) was greater in chow fed BBn rats, in comparison to BBdp rats. Despite the lower nutrient density of the chow diet (compared to casein), the higher fiber content of chow may stimulate greater intestinal proglucagon levels and increased nutrient transport capacity as reported in chow fed BBn rats (Reimer et al. 1998). This may reflect a mechanism of adaptation to high carbohydrate, higher fiber diets that is present in BBn rats, which does not appear to occur in BBdp animals at least at 30 days of age. Fiber source and solubility has also been reported to influence the gut of Wistar rats (Cavaglieri-Felippe et al. 1997), but may also be in response to an altered volume of food intake. Fiber viscosity has also been reported as an important factor in intestinal

response to dietary fiber (Gee et al. 1996). Massimino et al. (in press) have reported increases in jejunal villus height with high fermentable fiber feeding, in addition to increased expression of intestinal proglucagon mRNA, and intestinal glucose transporters. This may indicate the specific response of the intestine to dietary fiber. The mediation of intestinal trophism with fermentable fiber consumption may in part be occurring through the actions of the fermentation products of fiber, short-chain fatty acids (SCFA) (Koruda et a. 1988, Tappenden et al. 1996). Additionally, dietary fiber may impact the diabetic or pre-diabetic status of animals via GLP-1, a proglucagon fragment and insulin secretagogue (Holst 1994). Chow feeding is associated with increases in plasma GLP-1 (Reimer et al. 1997), which may increase β-cell stress by increasing insulin secretion.

The chow diet contains a greater variety of fiber sources than the casein diet (non-nutritive cellulose fiber source), including corn, wheat and soy. These have fermentabilities ranging from 45-80% after a 24 hour incubation with anaerobic intestinal bacteria (Van Soest et al. 1983, McBurney et al. 1989). Non-nutritive cellulose, as in the casein diet has a much lower fermentability; alphacel has been reported to have less than 5% fermentability after a 24 hour incubation with anaerobic intestinal bacteria (McBurney et al. 1989). These dietary factors may influence the responses seen in intestinal mucosa, as functional studies of the affects of these diets upon the intestine have reported changes in glucose transport and the presence of proglucagon (Reimer et al. 1998). Further, the potential of increasing pancreatic stress by inducing greater GLP-1 expression, as seen in studies by Reimer et al. 1997, may ultimately contribute to the reported high incidence of diabetes in chow fed rats (Scott et al. 1985, Hoorfar et al. 1992).

Other factors may have contributed to the intestinal response to diet. Specific nutrients may trigger or enhance pre-existing mucosal adaptive mechanisms (Ziegler et al. 1996, Tsai et al. 1997). Many trophic factors have been identified as contributing to this adaptive response. However, the effect of dietary nutrients on many of these factors is not known. Immunological factors, such as IL-11 (Fiore et al. 1998, Liu et al. 1996), and T cell activation via IL-2/IL-2R (Thompson et al. 1996) have been reported to affect intestinal trophism. Other elements, such as neurotransmitters and neuropeptides, as in

the case of neurotensin (López et al. 1997); exocrine factors (Altmann 1972), and the enteroglucagon active components glicentin (Myojo et al. 1997) and GLP-2 (Tsai et al. 1997) are reported to alter intestinal growth and adaptation. Additionally, epidermal growth factor (EGF) (Goodlad et al. 1985, Gasslander et al. 1997) has been suggested as a potential mediator of intestinal adaptation. The combination of nutrients such as glutamine in combination with trophic factors, such as insulin-like growth factor (IGF-1), can modulate intestinal adaptation (Ziegler et al. 1996). The importance of immunological input into gastrointestinal development is indirectly demonstrated by the observation that the immunosuppressive agent cyclosporin A administration delays the development of the gut, both morphologically and immunologically (Cummins et al. 1989).

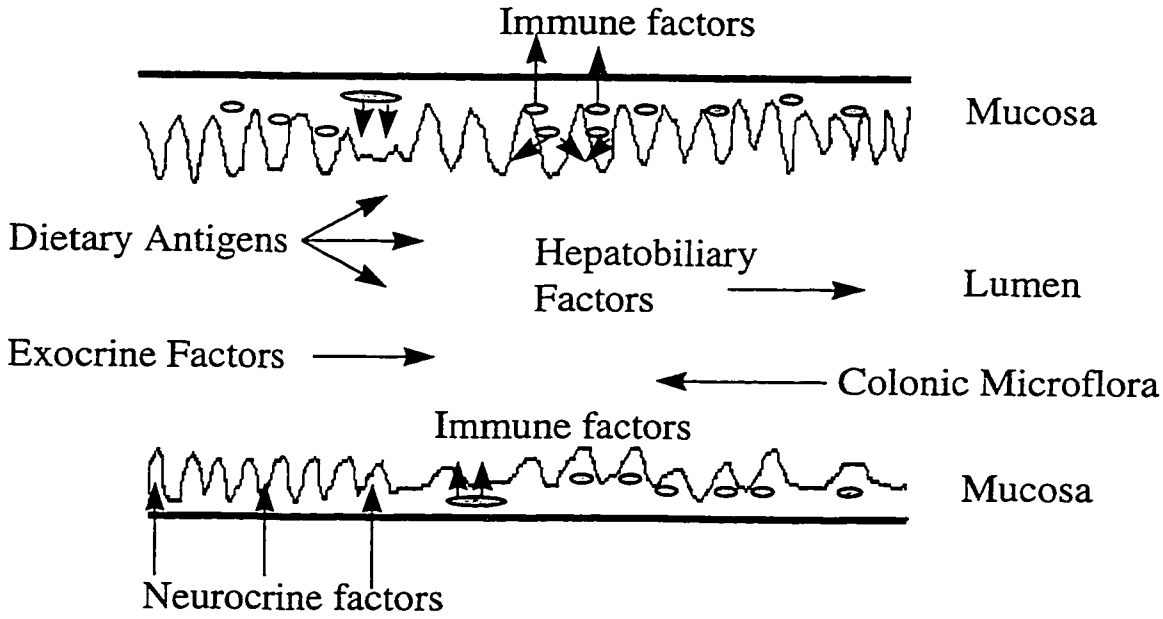


Figure 9. Schematic of factors influencing intestinal development, function and GALT. A large number of specific factors (indicated only by general subheadings of influential determinants) have been indicated to impact various attributes and capacities of the intestinal tract, with respect to adaptation, immunological parameters and development. The potential for interaction between the factors indicated above remains exponential. The gut represents a dynamic region and a potential target for a larger body of cross-communication between components, which exist both within, and outside of the intestinal tract.

In examining the influences of diet upon the morphology of the small intestinal mucosa two factors became increasingly clear. The first is that maternal diet has the capacity to modify the mucosal structure of the offspring, was not expected. The second reinforces the concept that changes occur during weaning and these can be modified by diet. The intestinal response to diet (via the dam) prior to weaning was similar in both BBdp and BBn rats, with a higher mucosal and villus height observed in pups from chow fed dams compared to pups from casein fed dams. After weaning, BBdp rats demonstrated a pattern of intestinal ontogeny unlike those of BBn rats.

Intestinal IgA Presence

It was hypothesized that the appearance and distribution of IgA bearing immunocytes would vary with BB strain and weaning diet. As hypothesized, chow feeding resulted in a higher number of IgA bearing cells in the jejunum compared to casein fed rats. The chow fed BBdp rats expressed significantly higher levels of IgA bearing cells than all other groups. There is little research available documenting intestinal IgA production in diabetes, however, several human studies have reported either absences or abnormally high levels of systemic IgA in individuals with diabetes (Smith et al. 1982, Hoddinott et al. 1982, Rodrigeuz-Segade et al. 1996, Gorus et al. 1998). We believe that the demonstration of higher levels of IgA bearing cells in rats fed the high incidence diet is novel.

IgA bearing cells were not detected in 15 day old animals in any group. Some non-distinctive staining was found in random samples, a pattern not seen in 30 day old animals or with other immunological marker staining in 15 day old animals. The presence of IgA bearing immunocytes has been reported to be initially detected on or about 15 days of age in rodent models, and the numbers of IgA bearing cells increases significantly at weaning (Crabbe et al. 1970). Thus, 15 days of age may have been too early to detect the presence of IgA bearing cells, and the small amount of staining may simply reflect IgA received through diet which may represent the initiation of IgA production, or the presence of maternal or hepatic secretory IgA.

Using antibodies in immunohistochemical studies, positive staining can identify only the presence of immune markers. This technique does not allow measurement of

direct production or secretion of an immunoglobulin (unless associated development and location markers are co-identified). Thus, changes in intestinal IgA production, detected by immunohistochemical techniques, may be the result of several factors. IgA is produced in response to a cascade of cytokines (Brandtzaeg 1996); thus altered T cell production of cytokines or altered signaling could trigger altered IgA production. Additionally, immune abnormalities in the BBdp rat (Field 1995, Yale et al. 1985) may contribute to altered IgA synthesis. Furthermore, because the primary synthesis of IgA and plasma cell maturation occurs at the liver in rats (cells that will ultimately populate the intestine), exposure at of immune cells to antigens in the liver may ultimately influence IgA production (Thomas et al. 1976). Further, as diet modulates hepatic function (Young et al. 1993), it may also indirectly impact IgA production or secretion. The measure of IgA secretion is complex; in humans, measures are limited to salivary or serum collections (Forest 1988, Forest 1992), or fecal sampling (Jiang et al. 1991), allowing only an estimation of intestinal IgA production. In experimental animals, the estimation of IgA secretion can be extended from these tissues to secretagogue-stimulate intestinal lavage (Elson et al. 1984) and flushings from the intestine (Merchant et al. 1991).

At birth, GALT is immature (Lyscom et al. 1983) and mucosal immune function and regulation is not fully developed (Brandtzaeg et al. 1991, Holt 1995). Excessive exposure of the neonatal immune system to antigens may predispose infants to gastrointestinal disease (Walker 1974). Human infants are born with little endogenous secretory immune capacity, and several months of development are required before it is functional (Brandtzaeg et al. 1991). Thus, there is a dependence on exogenous secretory factors, which are typically provided in breast milk. Genetic predisposition to diabetes and a short duration of breast feeding has been associated with the development of Type 1 diabetes in epidemiological studies (Gerstein 1994). Secretory immune defense is critical in the mediation of "immune exclusion" (Tomasi 1983, Childers et al. 1989), and aids in reducing systemic pathogen and environmental challenges in the neonate. Reduced IgA production (or IgA immunodeficiency) is associated with an increased risk of mucosal infection (gastrointestinal, respiratory) (Ammann et al. 1971). Viral infection in rats has been reported to induce diabetes in BBdp rats (Like et al. 1991, Crisa et al.

1992, Berdanier 1995) and BBn (Like et al. 1991) rats. Further, mucosal infection may trigger altered immune reactivity and increased susceptibility to autoimmune conditions.

Feeding the diet associated with a high incidence of diabetes in BB rats was associated with increases in the presence of IgA bearing cells. This suggests an effect of early diet upon immune function in diabetes susceptible animals. There are several possible explanations for increased numbers of IgA bearing cells. Chow is a multi-ingredient diet that contains a greater variety of potential antigens that may have stimulated IgA expression and production in diabetes prone BB rats. Increased antigen presence, increased systemic antigen uptake, and ultimately increased basolateral antigen exposure would trigger increase IgA production. Secondly, hepatic exposure to dietary antigens in rats may increase migration of lymphocytes reactive to dietary antigens, particularly if there is an increased uptake or absorption of antigens in the gut. It has been suggested that chow contains a potential diabetogenic antigen(s) such as cows milk protein (Scott 1994). Thirdly, enhanced IgA production in BB rats that are exposed to chow may also point to a defect the epithelium in the intestine. Such a defect has been reported streptozotocin-diabetic rats (Zoubi et al. 1995), with a significant increase in the number of epithelial cells, and altered crypt cell shape.

Dietary modulation of intestinal and systemic IgA presence has been studied under two conditions. The first, in experimental animals, suggests that protein-malnutrition decreases IgA concentration in small intestinal fluids (Sullivan et al. 1993, McGee et al. 1988). The second, suggests that infant feeding practices can alter salivary IgA (Gleeson et al. 1986). The finding of increased numbers of IgA bearing cells in chow fed BBdp rats indicates the potential for dietary ingredients, possibly proteins to modify intestinal IgA presence. Interestingly, dietary fiber may also alter IgA presence in the gut, as it able to alter parameters of T cell function at GALT (Field (b) et al. submitted).

IL-2 Receptor

It was hypothesized that the relative proportion of IL-2R bearing immunocytes would increase with chow feeding and vary with BB strain. The expression of lamina propria IL-2R did not vary between dietary treatments and BB strain in the present study. However, it was noted that high background immunostaining in BB rats may have

interfered with quantification of receptor expression.

The IL-2R acts to bind soluble IL-2, important in the propagation of immune response and immune cell activation (Hassan et al. 1996). Several forms of the IL-2 receptor are expressed; in these experiments, the antibody used is specific for the alpha chain on the activated IL-2R. Thus, cells must be activated to express this form of the receptor. Thus not all forms of IL-2R might have been detected in these animals. Additionally, the high affinity alpha chain (Rubin et al. 1990) is frequently released as soluble IL-2R into serum (Rubin et al. 1985), and therefore would not be detectable on the surface of cells in the gut.

Expressing the IL-2R bearing cell counts as a function of intestine (jejunal) length and total mucosal height may also provide some insight into the prevalence of this immune marker. Rough calculations suggest the greatest presence of IL-2R bearing cells occurs in chow fed BBn rats, when this measure is expressed per micrometer of jejunal length (roughly calculated as one half of the jejunoileum length). This assumes a constant expression of IL-2R bearing cells along the longitudinal axis of the gut, and confluent mucosa (as a rough basis). Thus, by this method, chow fed BBn rats expressed roughly 98 cells/cm; BBdp 65 cells/cm; casein fed BBn rats expressed 41 cells/cm, and BBdp 59 cells/cm. This may indicate a spatial expression of IL-2R not indicated by unadjusted immunohistochemical data; and would reflect the nature of IL-2/IL-2R involvement in intestinal trophism (Thompson et al. 1996) and may in part suggest a factor in the presence of the greatest mucosal measures in chow fed BBn rats.

Traditionally, BBdp rats display decreased responses to mitogens, *in vitro*, both at diabetes onset, and prior to overt disease (Yale et al. 1986, Field et al. 1990, Field et al. 1991). Although the neonatal BB rat immune system can be considered immature, T cells are able to produce IL-2 at both 15 and 30 days of age, (Field (a) et al. submitted). Altered response to mitogens in BB rats (Yale et al. 1986, Field et al. 1990, Field et al. 1991), low levels of IL-2 production in BBdp rats (Field (a) et al. submitted) and lymphopenia may complicate the detection of IL-2R in BBdp rats. Overcoming altered mitogen response in BB lymphocytes (such that activation may be detected) may require the administration or evaluation of a mitogenic factor other than dietary antigen. As in *in vitro* studies (Field 1995), administering mitogens with varying mechanisms of activation

improves lymphocyte response in BB rats. Thus, prior mitogen or antigen stimulation of intestinal samples may in turn be reflected in levels of IL-2R above those seen in these experiments with basal dietary stimulation.

The indication that immunohistochemically detectable IL-2R may be lower in BBdp rats may be a manifestation of lymphopenia, associated with diabetes prone rats. Lymphopenia can be detected as early as within the first two weeks of life in BBdp rats (van Rees et al. 1988b, Field (a) et al. submitted). Splenocyte IL-2 production was found to be lower in BBdp rats (at 15 and 30 days of age) regardless of chow or semi-purified diet feeding, as compared to BBn rats (Field (a) et al. submitted). The high level of background expression seen in chow fed, BBdp rats may indicate the expression or interference of factors which bind secondary antibodies (in this case, potentially cross-reactive to mouse IgG). Speculated binding agents could include higher levels of Fc receptors upon BBdp lymphocytes/tissues, or the expression of a protein with sequence overlap to an antigen being expressed in rat tissues. However, this phenomenon is not limited to immunohistochemical methods in BBdp rats (Field, pers. comm.).

Diet, Antigen Exposure and Diabetes

Intestinal immune development is known be modulated by several primary factors: intestinal exposure to viral and bacterial antigens, weaning, genetics and diet. Exposure to lumenal bacteria and viruses is required to initiate the development of immune structures and reduce intestinal permeability, as demonstrated in animals that are raised under germ-free conditions (Crabbe et al. 1968). Delayed weaning, or extended feeding with maternal milk have been shown (Van der Heijden et al. 1990) to retard intestinal immunological development, including IgA production. Thus, these combined factors prevent complete intestinal maturation prior to weaning. Further, maternal diet composition, likely via breast milk composition or immune factors (Brandorf 1980, Read et al. 1965) also impacts on intestinal development and may have influenced the expression of IgA bearing cells (at 30 days of age), as shown in the present study. Feeding the chow diet to genetically susceptible BB rats accelerates or alters intestinal development and increase IgA expression, which may be related to diabetes development.

In these experiments we have demonstrated the potential of two diets known to

modulate diabetes incidence and onset to modulate intestinal and immune parameters. We have also shown that after weaning, BBdp rats respond to diet in a manner different to weaned BBn rats. This further indicates the critical changes associated with weaning, and suggests a mechanism by which diet may modulate diabetes incidence.

Future Directions in Research

Understanding the mechanisms of Type 1 diabetes pathogenesis increases the potential for interventions and for therapeutic interventions. Many of the mechanisms behind the interaction of genetic susceptibility and environmental factors remain to be elucidated. Using this animal model, there are several specific questions to be addressed. Altered lamina propria IgA presence may be indicative of basolateral antigenic exposure, potentially due to a compromised epithelial barrier. Thus, testing epithelial integrity in response to antigen challenge and electrical conductivity may further indicate the influences of diet and diabetes susceptibility upon barrier function. Reduced barrier integrity may suggest increased interaction with environmental antigens, such those from viral and dietary sources.

Defining specific immunological adaptation to diet in the BB rat model, such as the study of pancreatic infiltrating lymphocyte cytokine production in response to chow and casein feeding (Scott et al. 1997) may further the understanding of how these diets are able to influence diabetes susceptibility. This work suggests casein feeding has the capacity to induce greater levels of Th2 cytokine production in the pancreas (Scott et al. 1997). Th2 cytokine production is believed to be a protective influenced in autoimmune disease (Powrie et al. 1993, Romagnani 1992). Kleeman et al. 1998 have demonstrated the capacity for dietary fat to alter cytokine profiles of immunocytes within the gut of BB rats. Altering cytokine profiles at the pancreas and gut through diet may then have the capacity to alter other immune parameters at these sites. Thus, further studies of intestinal immunity in the BB rat could examine specific immune tissues within the gut with dietary modulation; including intraepithelial lymphocytes, epithelial barrier integrity, and the role of diet in oral tolerance induction.

Examining chow fed BBdp rats at time points closer to weaning, perhaps at 20 and 25 days of age, would aid in identifying the critical point at which diet alters intestinal structure and the expression of IgA bearing cells in chow fed BBdp rats.

Knowing now that the intestinal mucosa of pups seems to respond to maternal nutrition at 15 days of age, and that this 'adaptive' response is not present at 30 days of age narrows down the critical time frame more closely to weaning itself. This may further the collective understanding of the critical association of weaning and diabetes susceptibility. Also, as in previous work (Scott et al. 1988, Hoorfar et al. 1991), the search for a specific dietary component which has the capacity to induce diabetes or immunological modulatory properties may be of importance to the elucidation of dietary influence in the pathogenesis of diabetes. The addition of isolated constituents from chow to the casein diet on gut structure and immune function may further identify the 'diabetogenic substance(s)' in chow.

Further, as the production of IgA in the rat is primarily controlled at the level of the liver, examining more closely the role of the liver as an immunological organ in this model may further reveal involvement in dietary adaptation or immunological function. Other candidates for study include the role of psyschoneural-immune and neural-gastrointestinal interactions in immunity. Examining non-traditional immune and endocrine affectors, such as prolactin (Ellis et al. 1997) may indicate other factors involved in gastrointestinal immune function. Also, the link between diet, intestinal immunity and autoimmunity, as seen in the capacity of diet to modulate pre-diabetes intestinal status in this model, requires further examination for potential therapeutic avenues.

The functional nature of IgA responses, its critical role in regulating antigen exposure and its association with tolerance induction and dietary adaptation, (as seen in the present study) may aid in the understanding of development of oral tolerance. This in turn may further exhibit relationships between the induction or abrogation of tolerance and the development of autoimmune conditions and allergy, as well as the effects of early antigen exposure.

The present study has indicated the potential for early diet to alter both intestinal mucosal development and intestinal immunity. Further, these experiments have indicated the interaction of diet with intestinal structure and immunity prior to the onset of diabetes. These findings suggest and reinforce several significant concepts, including the role of diet and the link between a component of intestinal immunity. The development

of the neonatal BB rat small intestinal mucosa can be modulated by diet. The presence of IgA bearing cells in the small intestine can also be altered by dietary modulation. In combination, these findings suggest that diets, which alter diabetes incidence in BB rats, are able to modify intestinal structure and intestinal immunity in very young BB rats. These findings suggest a critical link between intestinal changes and diet, which are paralleled in the literature by diabetes susceptibility in adult animals.

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Appendix 1: Solution composition.

Harris Hematoxylin

Hematoxylin (*Fisher Scientific,* Fairlawn, New Jersey, USA)	2.5g
Absolute ethanol	25mL
Distilled water	500mL
Mercuric oxide	1.25g
Potassium alum	50g

Dissolve hematoxylin in absolute ethanol. Add alum (previously dissolved in hot water in a 2L flask). Bring rapidly to a boil, and observe until solution turn dark purple. Rapidly cool solution (into ice water). Add glacial acetic acid. Filter before use. Note: The addition of mercuric oxide may cause explosive reaction, therefore, solution should be prepared in a large flask and fume hood.

Eosin

Eosin Y (C.I. 45380) (*Fisher Scientific*, Fairlawn, New Jersey, USA)	1.0g
70% (v/v) ethanol	1000.0mL
glacial acetic acid	5.0mL

Scott's Tap Water

sodium bicarbonate	2.0g
magnesium sulfate	20.0g
distilled water	1000.0mL

Appendix 2, Table 1. Examination of the reproducibility of immunostaining cell quantification.

Slide Number	Count 1 (cells/villus)	Count 2 (cells/villus)
741	1.4	1.2
053	1.7	1.8
923	2.6	2.2
322	1.1	1.2
T181	0.7	0.4
585	1.1	0.7
725	0.2	0.7
227	2.3	2.1
314	0.4	0.3
114	0.1	0.3
043	1.1	0.8
143	0.3	0.3

A random selection slides were chosen for re-counts of positively stained cells. A two-tailed T-test indicated the counts were not different ($p \leq 0.3$).

Appendix 3. Images of immunohistochemical staining. Images presented are those at magnification of 100X.

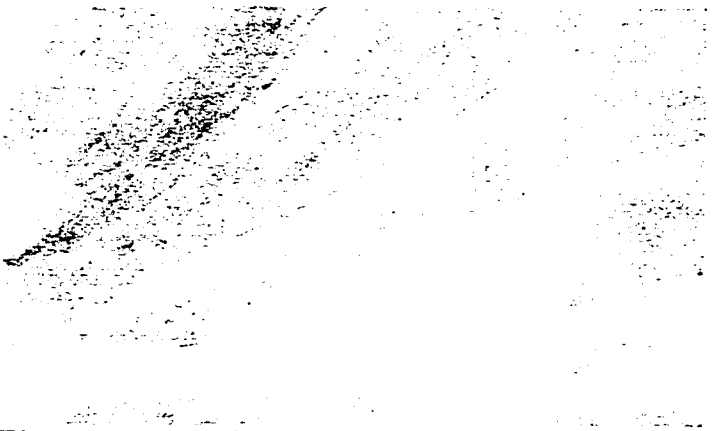

Figure 1. Jejunal section of a chow fed BBn rat at 30 days of age with no primary antibody application (CONTROL).

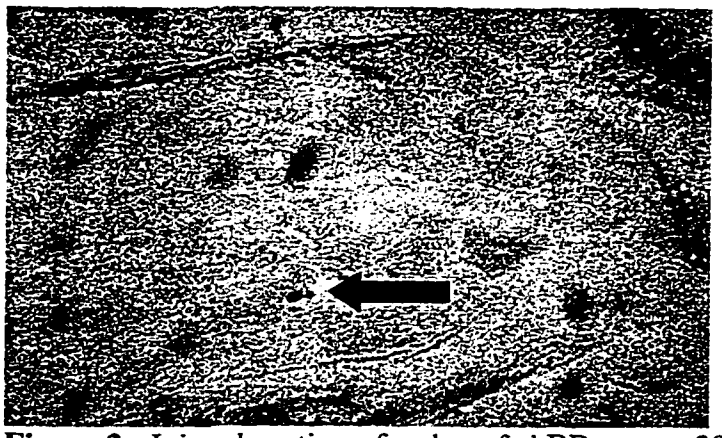

Figure 2. Jejunal section of a chow fed BBn rat at 30 days of age with IgA staining.

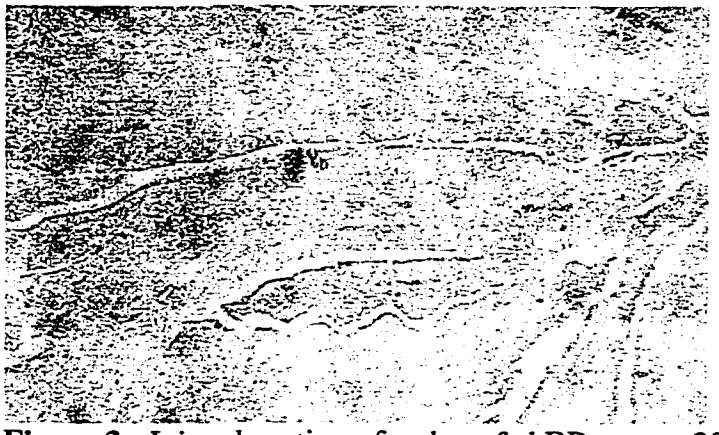

Figure 3. Jejunal section of a chow fed BBn rat at 30 days of age with IL-2R staining.

Figure 4. Jejunal section of a chow fed BBdp rat at 30 days of age (CONTROL).

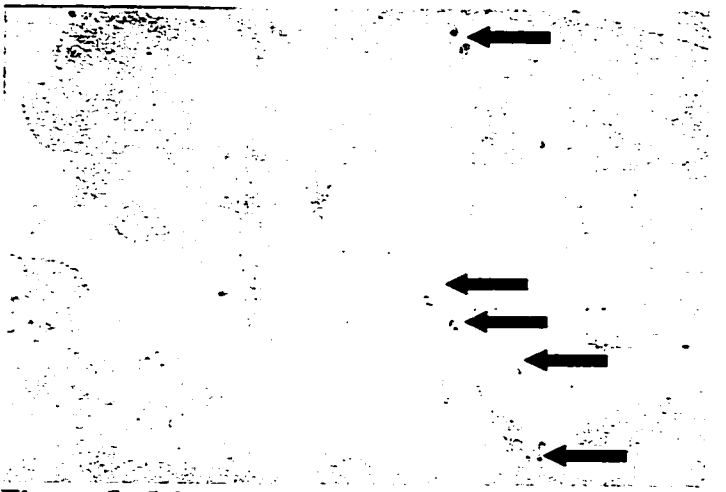

Figure 5. Jejunal section of a chow fed BBdp rat at 30 days of age with IgA staining.

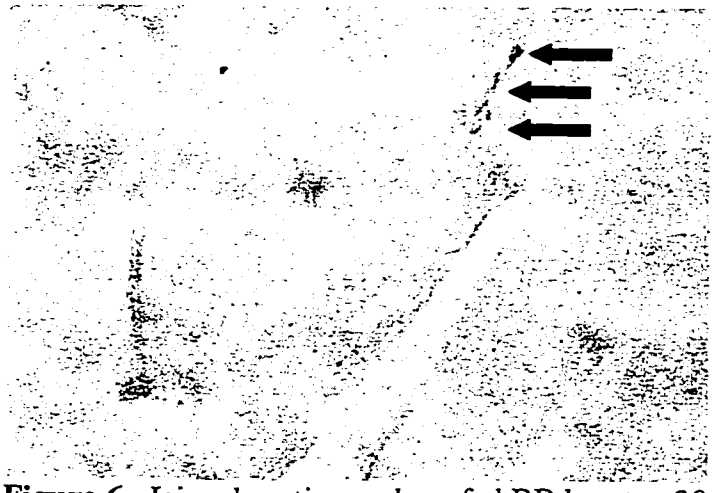

Figure 6. Jejunal section a chow fed BBdp rat at 30 days of age with IL-2R staining.

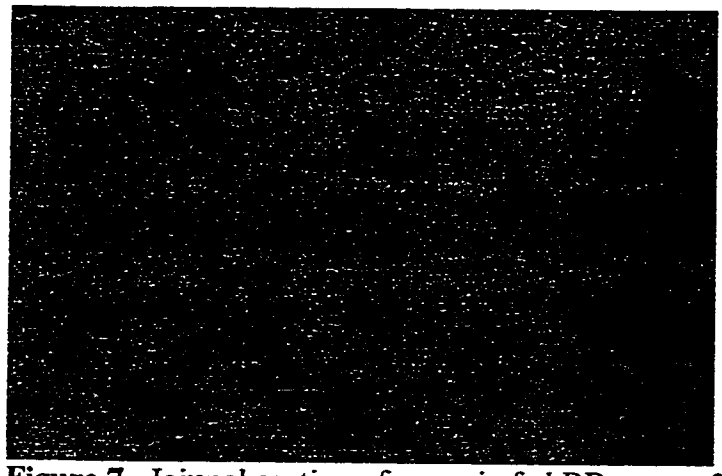

Figure 7. Jejunal section of a casein fed BBn rat at 30 days of age (CONTROL).

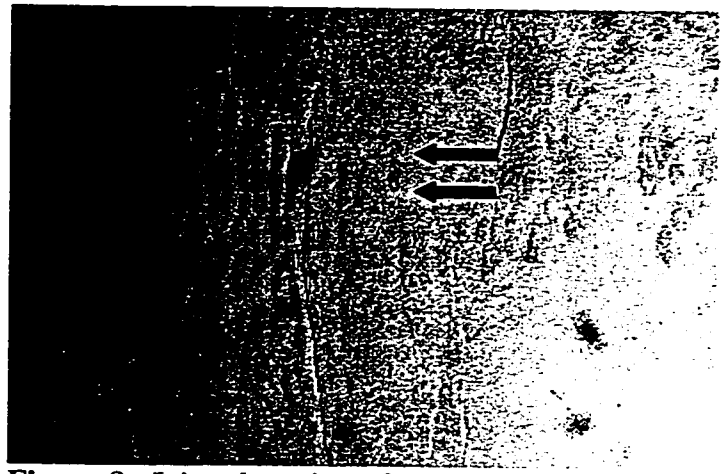

Figure 8. Jejunal section of a casein fed BBn rat at 30 days of age with IgA staining.

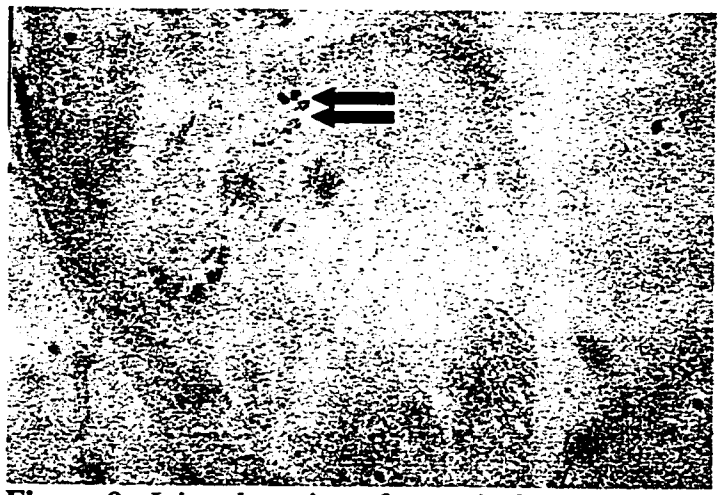

Figure 9. Jejunal section of a casein fed BBn rat at 30 days of age with IL-2R staining.

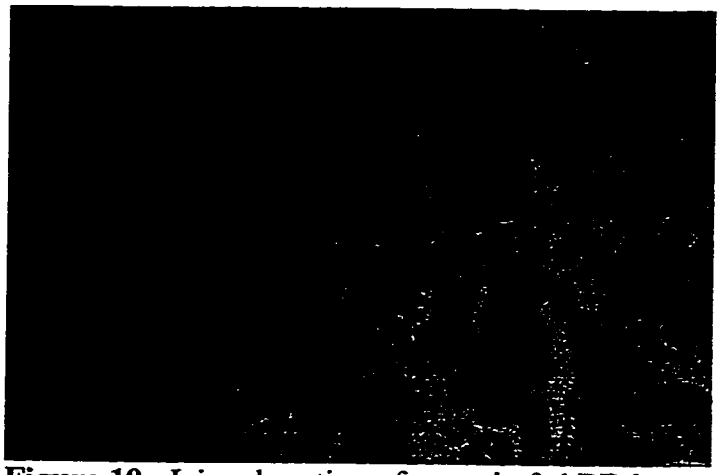

Figure 10. Jejunal section of a casein fed BBdp rat at 30 days of age (CONTROL).

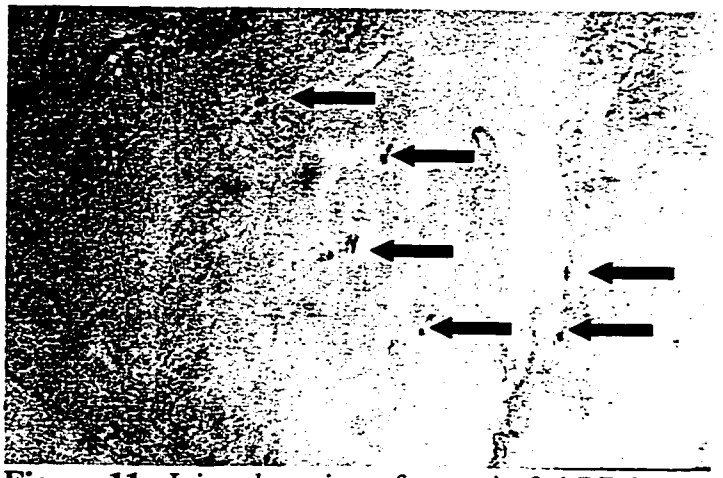

Figure 11. Jejunal section of a casein fed BBdp rat at 30 days of age with IgA staining.

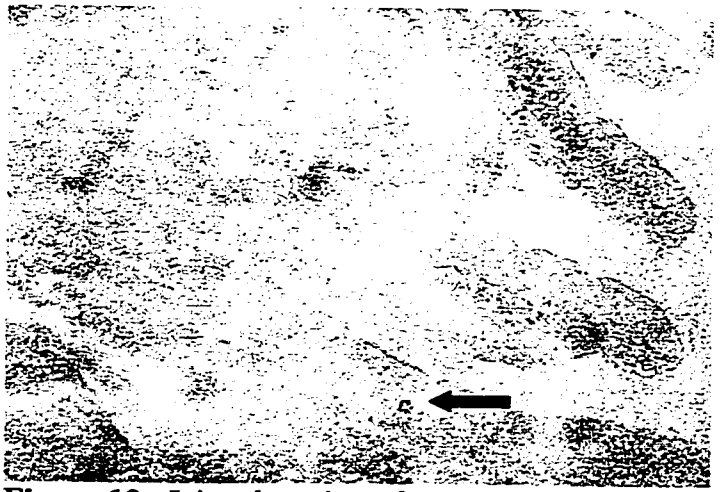

Figure 12. Jejunal section of a casein fed BBdp rat at 30 days of age with IL-2R staining.

Figure 13. Jejunal section of a 15 day old rat (CONTROL).

Figure 14. Jejunal section of a 15 day old rat with IgA staining (non-distinctive regional staining).

Figure 15. Jejunal section of a 15 day old rat with non-apparent IgA staining.

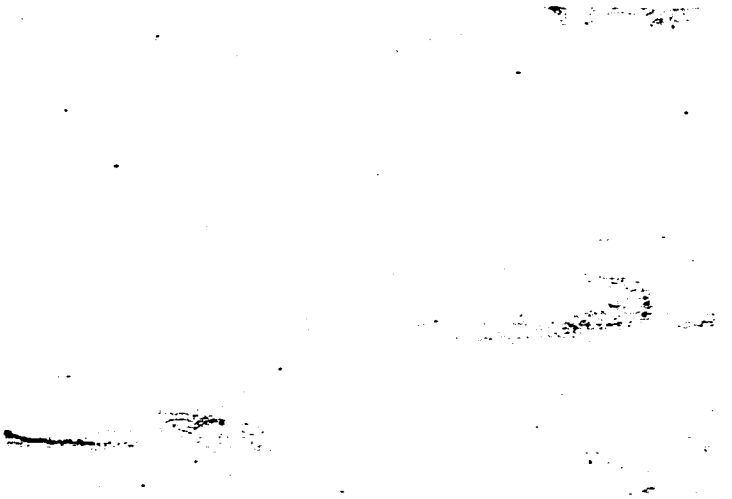

Figure 16. Jejunal section of a 15 day old rat with non-apparent IL-2R staining.

Figure 17. Jejunal section of a 15 day old rat with no staining for αβ TCR staining (exemplifies no staining of γδ TCR staining as well).