AN IMMUNOMODULATING MYCOTOXIN INTERFERES WITH THE DEVELOPMENT OF AUTOIMMUNE DIABETES IN DIABETES-PRONE BB/WOR RATS

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To my parents,
my wife and my son

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Mycotoxins are structurally diverse secondary metabolites of fungi that are produced when fungal pathogens infect human or animal tissues; mytotoxins may also be ingested with fungus contaminated food. The clinical syndromes caused by fungal toxins have been extensively characterized and range from acute to chronic diseases with mild symptoms to fatal outcomes. Some mycotoxins, at levels that do not cause overt clinical mycotoxicosis, suppress immune functions and may decrease resistance to infectious disease. The sensitivity of the immune system to mycotoxin-induced immunosuppression arises from the vulnerability of the cells that participate in immune mediated activities and regulate the complex communication network between cellular and humoral components. Although the cellular-molecular basis for many of the specific immunosuppressive effects of mycotoxins is unclear, inhibition of DNA, RNA and protein synthesis by means of various mechanisms appears to be directly or indirectly responsible for the immunosuppressive action of many mycotoxins (Cusumano, et al., 1996; Thuvander et al.,1996).

Gliotoxin (GT) is one such fungal secondary metabolite that reduces host resistance to *Aspergillus* and *Candida* infections in humans, and functions as an immunomodulator. It has been proposed that the effect of GT on the immune system may serve to make certain fungal infections more chronic. In addition to its function as a virulence factor, it may have a useful role as an immunologic reagent. Although GT has been used to inhibit transplantation rejection (Mullbacher,1984; Mullbacher, et al., 1986; Waring and Mullbacher, 1992), the research reported in this dissertation is the first study in which GT is used to prevent autoimmune insulin dependent diabetes mellitus (IDDM).

Insulin Dependent Diabetes Mellitus

Autoimmune disorders

Autoimmune diseases are disorders caused by inflammation and destruction of tissues by the body's own immune actions. Autoimmune diseases develop on a susceptible background involving multiple genes (Davies, 1994), some of which are associated with immune regulation and some of which control target-organ functions (Kennedy, German and, Rutter, 1995; Bennett, et al., 1995). Although the mechanism is not clear and may differ with the specific autoimmune disease, several hypotheses have been proposed to explain the autoimmune diseases (Theofilopoulos, 1995). The poorly displayed "cryptic self" determinants allow autoreactive lymphocytes to escape from negative selection. Faulty antigen presentation stimulates autoimmunity by presenting self antigens. Molecular mimicry has been reported to result in cross reaction between self and foreign peptides. Autoimmunity may also arise as a result of immune responses against modified self-determinants. Errors in central and peripheral tolerance at the T and B lymphocyte level are also implicated as causes for autoimmunity. Polyclonal B and/or T cell activation by superantigens has been considered a contributing or initiating mechanism in autoimmunity, particularly in systemic autoimmune diseases. Although these various sources of autoimmunity may account for some cases, most investigators consider immunoregulatory disturbances as a primary cause of autoimmune diseases. Some T lymphocyte subsets, such as $CD4^+$, $CD8^+$ and $RT6^+$ T cells, are involved in the regulatory process. A changing balance between Th1 and Th2 lymphocytes provides a good example of autoimmune induction through immunoregulatory disturbance.

In humans, autoimmune reactions might reflect immune defects such as occur in lymphoproliferative disorders (Brouet, Mariette and Sligmann, 1994), in patients carrying null complement alleles (Batchelor, et al., 1987), Fas mutations (Rieux-Laucat, et al., 1995; Fisher, et al., 1995), or in chronic graft-versus-host disease (Goldman, Druet and Gleichmann, 1991). Other uncommon situations include autoimmune paraneoplastic syndromes (Lennon, et al., 1995) and pregnancy (Kelly, et al., 1988), in which tumor cells and the placenta, respectively, express autoantigens shared by a peripheral tissue. Autoimmunity may also be induced by infectious agents (Kotb, 1995) or drugs (Goldman, Druet and Gleichmann, 1991) that modify the tissue and stimulate the immune system.

There are two categories of autoimmune diseases, organ-specific and systemic autoimmunity. The autoantibodies of organ-specific autoimmune disease are associated with components of the specific organ so that a lesion or target tissue destruction is localized. The organ-specific diseases involve the thyroid (Hashimoto's thyroiditis, primary myxoedema and thyrotoxicosis), the stomach (pernicious anaemia), the adrenal (Addison's disease), or the pancreas (IDDM). The organ-specific diseases are caused by humoral immunological responses against self-antigens for which T-cell tolerance is normally not established.

The immune responses of systemic autoimmune diseases are directed against distributed antigens. Although systemic autoimmune damage is widely dispersed, certain organs or tissues may be particularly affected by systemic autoimmunity. For example, the systemic autoimmune diseases include dermatomyositis of muscle, systemic lupus erythematosus (SLE) of joint, skin and kidney, scleroderma of skin and kidney, and

rheumatoid arthritis of joints. The causes of systemic autoimmune diseases are less clear than the tissue-specific autoimmune diseases. Gene defects leading to inadequate T cell tolerance may explain some cases of systemic autoimmunity. Defective of apoptosis, for example, is the mechanism in murine lpr/gld SLE models (Theofilopoulos, 1995).

Target tissue of autoimmune diseases

The anatomical and physiological organization of target tissues influences many aspects of autoimmune diseases and their respective clinical manifestations. These include the homing of inflammatory cells and lymphocytes, antigen presentation, the level of autoantigen expression or expression of neoantigens, and the spread of the autoimmune reaction. Moreover, several features point to antigen-driven responses in autoimmunity, including the use of recurrent idiotypes and V genes, the high rate of replacement mutations in complementarity-determining regions (CDRs) of the variable domains of autoantibodies (Radic and Weigert, 1994), and the clustering of autoantibodies directed against antigens associated with subcellular particles in a particular disease (Tan, 1991). Genetic susceptibility conferred by genes encoding autoantigens (e.g., myelin basic protein, acetylcholine and thyrotropin receptor, and insulin) has also been reported in autoimmune diseases (Garchon, et al., 1993; Boylan, et al., 1990; Tienari, et al., 1992; Bell, Horita and Karam, 1984; Julier, et al., 1991). Additional evidence for the role of target cells includes demonstration of early target organ dysfunction in autoimmune conditions (Homo-Delarcher, Boitard and Boitard, 1996). The autoimmune antibodies appear and are consistently detected beginning at a very early stage of autoimmune disease

development. The autoimmune antibodies, while probably not being responsible for the majority of autoimmune damage, serve to make early diagnosis possible so that intervention strategies can be applied before onset of significant autoimmune damage.

Insulin dependent diabetes mellitus as an autoimmune disease

Diabetes mellitus comprises an etiologically and clinically heterogeneous group of hyperglycemic disorders. Hyperglycemia is the consequence of a relative or absolute deficiency of insulin in the presence of a relative or absolute excess of glucagon. When the insulin deficiency is extreme, these hormonal abnormalities are responsible for the tendency to develop ketoacidosis. Diabetes is associated with a set of late onset complications involving the eyes, kidneys, nerves, and blood vessels. Diabetes is fatal in the absence of insulin treatment which must be continued for life (Wilson and Foster, 1992). The term "type I diabetes mellitus" is used to specify diabetes secondary to autoimmune destruction of insulin producing pancreatic islet ß-cells. This disease is also known as insulin-dependent diabetes mellitus (IDDM) or juvenile onset diabetes since patients survive only with exogenous insulin treatments and the disease usually develops at an early age. IDDM is a disorder of carbohydrate metabolism in which glucose is not oxidized to produce energy due to a lack of insulin. In contrast, type II diabetes is due to peripheral resistance to insulin. IDDM develops in about three per 1,000 persons in the general population and in about three per 100 first-degree relatives of patients with type I diabetes (Eisenbarth, 1989). As indicated in Table 1, human IDDM is known to be associated with a recessive diabetogenic gene in the major histocompatibility complex

Table 1. Susceptibility loci and candidate genes for autoimmune diabetes (Theofolopoulos, 1995).

Chromosomal location	Locus designation	Candidate genes
NOD mouse		
1	Idd-5	Bcgl/Lch/Ity
3	Idd-3	IL-2
3	Idd-10	Fcgr1, Csfm, Cd53
4	Idd-9	-
6	Idd-6	Bphs
7	Idd-7	-
9	Idd-2	-
11	Idd-4	Nosi
14	Idd-8	-
17	Idd-1	MHC
BB rat		
4	Iddm1/Lyp	-
20	Iddm2	MHC
Human IDDM		
2q	-	GAD1
6p21	IDDM1	MHC
6q	IDDM5	SOD2
8p	-	-
8q	-	-
10q	-	GAD2
11q13	IDDM4	-
11p15	IDDM2	INS
18q	-	-

(MHC) on chromosome 6, although expression of a particular HLA gene is not by itself sufficient to cause autoimmune disease (Theofilopoulos, 1995). Both class I and class II MHC molecules seem to have a role in the immune reactions that characterize development of IDDM, although the mechanism is unknown.

The pancreatic islets of Langerhans are the targets of the autoimmune reaction that leads to IDDM. This highly selective destruction of 60-70% of the insulin-secreting β cells, the major endocrine component of the islet (Bach, 1994; Leahy, Bonner-Weir and Weir, 1988) leads to disease. Insulitis, the histological hallmark of IDDM, varies in severity from one islet to another in an individual pancreas. The disease in humans follows a highly chronic course and clinical diabetes is only manifest when a majority of β cells has been destroyed.

Bold experimental procedures cannot be undertaken in humans, making the availability of animal models of IDDM critical for diabetes research. Animal models include rats and mice. Rats seam relatively tolerant of pancreatic destruction, remaining normoglycemic following 60% pancreatectomy (Wilkin and Armitage, 1985). As in humans, a very substantial loss of pancreatic function is required for clinical diabetes. In the NOD mouse model of IDDM, insulitis precedes hyperglycemia by weeks or months; in humans, the detection of autoantibodies precedes diabetes onset by several years. Evidence of autoimmunity in IDDM includes the detection of both autoantibodies and T cells specific to islet autoantigens, as well as an association with a restricted set of MHC class II alleles.

Autoantigens

IDDM has been considered as an autoimmune disease because of the discovery of islet cell autoantibodies (ICAs) in patients with a recent onset of the disease (Beakkeskov, et al., 1982). Several autoantigens in IDDM that are recognized by autoantibodies have been studied for almost two decades (Table 2). The most commonly mentioned autoantigen in IDDM is a 64 kDa β-cell-specific glutamic acid decarboxylase (GAD). Circulating autoantibodies to GAD antigen are often detectable by immunoprecipitation of radiolabeled islet lysates with IDDM sera (Michelsen et al., 1991). GAD catalyzes the conversion of glutamic acid to γ-aminobutyric acid, which is the major membrane-stabilizing neurotransmitter of the central nervous system. Two forms of GAD, known as GAD65 and GAD67, are encoded by separate genes (Bu, et al., 1992; Robin, et al., 1994). An islet-surface autoantigen, ICA-512, has been described recently, which is a member of the protein tyrosine phosphatase (PTP) family (Lan, et al., 1994), and another ICA antigen has recently been identified and termed insulinoma antigen 2 (IA-2). The typical fragment of IA-2 is 40 kDa and its derivative, IA-2β, is 37 kDa. Insulin itself is also a β-cell-specific autoantigen (Song, Li and Maclaren, 1996). There are several other putative candidate autoantigens in IDDM that remain under investigation (Table 2). These autoantigens may be starting points of IDDM pathogenesis or may simply be early signs of IDDM. GAD can be detected by autoantibody in immunoprecipitation reactions long before clinical IDDM onset. The early detection of autoantibodies suggests that it may be possible to use them as an early warning to trigger clinical immune intervention to block the autoimmune process, or to use other therapeutic reagents to cure IDDM.

Table 2: Autoantigens and candidate autoantigens in IDDM (Song, 1996)

Research situations	Autoantigens
Atoantigens in IDDM	GAD65
	GAD67
	Tyrosine phosphatases IA-2 and IA-2β
	Insulin
Candidate autoantigens in IDDM	Insulin receptor
	Carboxypeptidase H
	Peripherin
	ICA-reactive gangliosides
	Heat shock protein 65 (Hsp65)
	ICA-69

Animal Models

General characteristics of DP/BB rats

For the study of IDDM, several useful animal models have been developed. These include the spontaneously diabetic BioBreeding (BB) rat and the NOD mouse models. Since this research used the BB rat model, most of the review of animal models will be devoted to BB rats. Characteristics of diabetes in the BB rat closely parallel those observed in human IDDM. The genetic, immunological and environmental factors that predispose to the disease can be investigated under controlled conditions. There are two inbred lines in this model system, diabetes prone (DP) and diabetes resistant (DR) BB rats, which were developed from the Wistar rat line. DP/BB rats were used exclusively in this research. DP/BB rats develop diabetes abruptly at 60-120 days of age with an incidence of about 86%. This condition is characterized by sudden weight loss, hyperglycemia, hypoinsulinemia, glycosuria and ketonuria. Histologic insulitis (lymphocyte infiltration of pancreatic islets) is observed at the onset of spontaneous diabetes mellitus in DP/BB rats. A venular defect characterized by lymphocyte adherence and loss of endothelial integrity in the pancreas has been described in both DP/BB and DR/BB rats. Autoimmune thyroiditis, an increased susceptibility to pulmonary infections, and other abnormalities have also been reported in DP/BB rats (Crisa, Mordes and Rossini, 1992; Guberski, 1994).

Cellular and molecular aspects of DP/BB rats

Unlike human IDDM, severe lymphopenia is a key feature in DP/BB rats, with a marked decrease of $CD4^+$ and $CD8^+$ T cell subtypes (Prud'homme et al., 1988; Jackson, 1983; Woda, et al., 1986), and relative increases of $CD5^+$ B cells and NK cells (Woda and Biron, 1986). $RT6^+$ cells (a subset of mature T lymphocytes described in detail below) are virtually undetectable in the spontaneously diabetic DP/BB rat. In contrast, normal numbers of T cells expressing the RT6 alloantigen are present in the periphery of DR/BB and other normal rat strains (Table 3).

Susceptibility loci in the diabetes prone BioBreeding (DP/BB) rat have been identified (Theofilopoulos, 1995). A locus, iddm2, is linked to the rat MHC on chromosome 20 and contributes to diabetes in a semidominant fashion. Unlike the NOD mouse and human IDDM, the DP/BB rat is characterized by a recessively inherited severe T-cell lymphopenia due to iddm1 located on chromosome 4 (Table 1).

Environmental factors

In addition to genetic predisposition, environmental factors are involved in the pathogenesis of IDDM in humans as well as experimental animals. Certain viruses such as rubella, coxsackievirus, and cytomegalovirus have been reported to play a role in human diabetes pathogenesis. Parvovirus and Kilham rat virus, are considered as triggers of IDDM in BB rats. In contrast, lymphocytic choriomeningitis (LCM) virus actually prevents IDDM in NOD mice and BB rats. Mouse hepatitis virus, encephalomyocarditis virus, and lactate dehydrogenase elevating viruses have been reported to prevent IDDM in

Table 3. Clinical and immunological iharacteristics of diabetes-prone and diabetes-resistant BB/Wor rats (Crisa, et al., 1992)

	Diabetes-prone	Diabetes-resistant
Cumulative frequency of diabetes	86%	< 1%
Age at onset	> 85% of cases between 55 and 120 days of age	usually between 50 and 60 days of age
Insulitis		
Diabetics	yes	yes
Nondiabetics	50%	13%
Thyroiditis	5-100% depending on subline	11%
Peripheral lymphocytes	lymphopenic	nonlymphopenic
Lymphocyte subsets		
OX19 (CD5)	low	normal
W3/25 (CD4)	low	normal
OX8 (CD8)	low	normal
OX22, OX32 (CD45R)	low	normal
RT6	absent or very low	normal
$RT6^{+}$ intraepithelial lymphocytes	present	present
NK cell activity	increased	normal
In vitro response of T cells to mitogen	depressed	normal

NOD mice. Some bacterial and fungal extracts have been reported to exert effects against IDDM in NOD mice and BB rats (Rabinovitch, 1994). Laboratory diets containing plant products, such as gluten flour and soybean meal, can enhance diabetes pathogenesis while semisynthetic diets rich in L-amino acids and hydrolyzed casein diets reduce the incidence and delay the onset of diabetes (Crisa, Mordes and Rossini, 1992; Guberski, 1994). Vitamin A intake is also involved in diabetes development. Retinol deficient diets decreased diabetes and insulitis in DP/BB rats and retinoic acid partly substituted for retinol in the development of insulitis. (Driscoll, et al., 1996)

Modification of Disease by Gliotoxin

Chemistry of gliotoxin

GT is a secondary fungal metabolite originally isolated in 1932 from a strain of *Trichoderma lignosum*. GT (Figure 1) was the first described member of the epipolythiodioxopiperazine (ETP) family (Figure 2) which comprises more than 50 separate compounds produced by the higher filamentous fungi. GT has antimicrobial activity, especially against gram-positive bacteria, and also inhibits the multiplication of some RNA viruses. Dose-dependent toxicity of ETP compounds against a variety of mammalian cells has been reported, although susceptibility varies with cell type. These diverse biological activities derive from a reactive disulfide bridge on the piperazine ring that may form mixed disulfides with sulfhydryl or disulfide groups on critical cellular proteins. This causes oxidation or reduction of cellular components, or may trigger surface

Figure 1. Molecular structure of gliotoxin (GT). GT is a fungal secondary metabolite and a member of the epipolythiodioxopiperazine (ETP) family. The disulfide group of GT which bridges the piperazine ringmay bind to disulfide groups on target proteins to trigger cell surface receptors or participate in oxidation-reduction cycling.

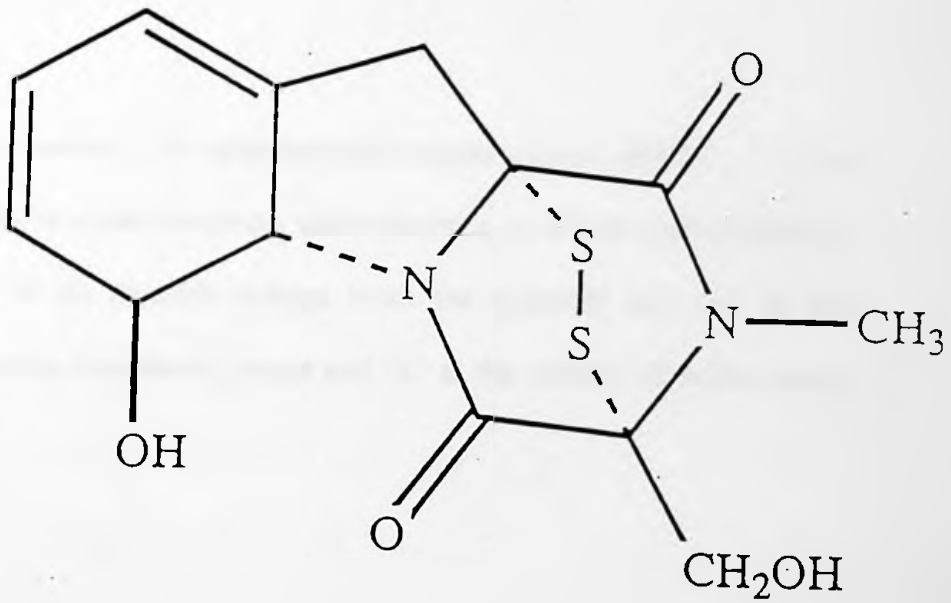
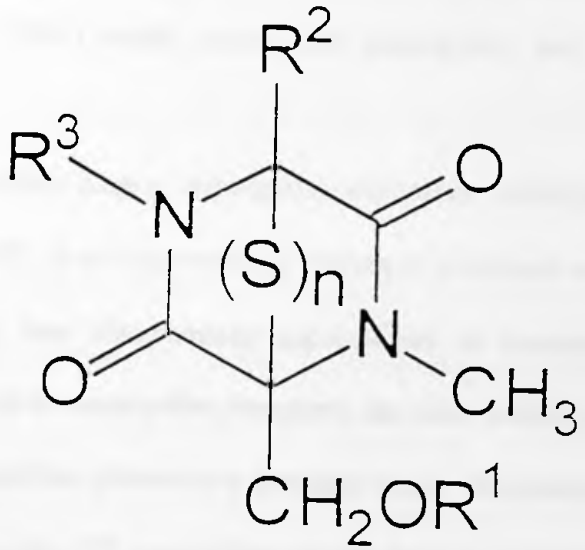

O
S
S
N
N
CH3
OH
O
CH2OH

Figure 2. General structure of epipolythiodioxopiperazines (EPT). The diketopiperazine ring is flat in crystal structure, interconverting to a boat conformation in solution. The presence of the disulfide linkage locks the molecule into one of the conformations. "R" indicates substituent groups and "n" is the number of sulfur atoms which range from 1 to 4.


R2
R3
N
O
(S)n
N
O
CH3
CH2OR1

receptors by cross-linking them through mixed disulfide linkages (Waring, Eichner and Mullbacher, 1988).

Biological effects of gliotoxin

GT, produced by fungi in the vertebrate host, could play a significant role in the pathogenesis and mortality of aspergillosis. The potential importance of GT is indicated by the observation that it is synthesized by many different fungi, including strains of *Candida* (Shah and Larsen, 1991) which, along with *Aspergillus*, are the two most prevalent pathogenic fungi.

A saprophytic, thermophilic fungus, *Aspergillus fumigatus*, which produces GT in culture (Mullbacher, et al, 1985), is an opportunistic pathogen in humans and animals and is responsible for potentially fatal disseminated aspergillosis in immunocompromised individuals. Individuals exposed to *Aspergillus fumigatus* can also display aspergilloma or "fungus ball" which is a saprophytic growth in a damaged lung. Mullbacher and Eichner (1984) have proposed that *in situ* GT production by the fungus *Aspergillus fumigatus* enhances the progression of lung disease.

Immunological effects of gliotoxin

GT has been described as an immunomodulator and possesses *in vitro* immunosuppressive activity. This compound inhibits phagocytosis by activated peritoneal macrophages (Waring, et al., 1990) and also inhibits mitogen-induced T cell proliferation and the activation of cytolytic T lymphocytes, by partially inhibiting lymphokine release

(Mullbacher, 1984; Mullbacher, et al., 1986). The *ex vivo* treatment of bone marrow with GT prevents graft-versus-host disease in allogeneic bone marrow transplantation by selectively depleting the bone marrow of mature lymphocytes (Waring and Mullbacher, 1992).

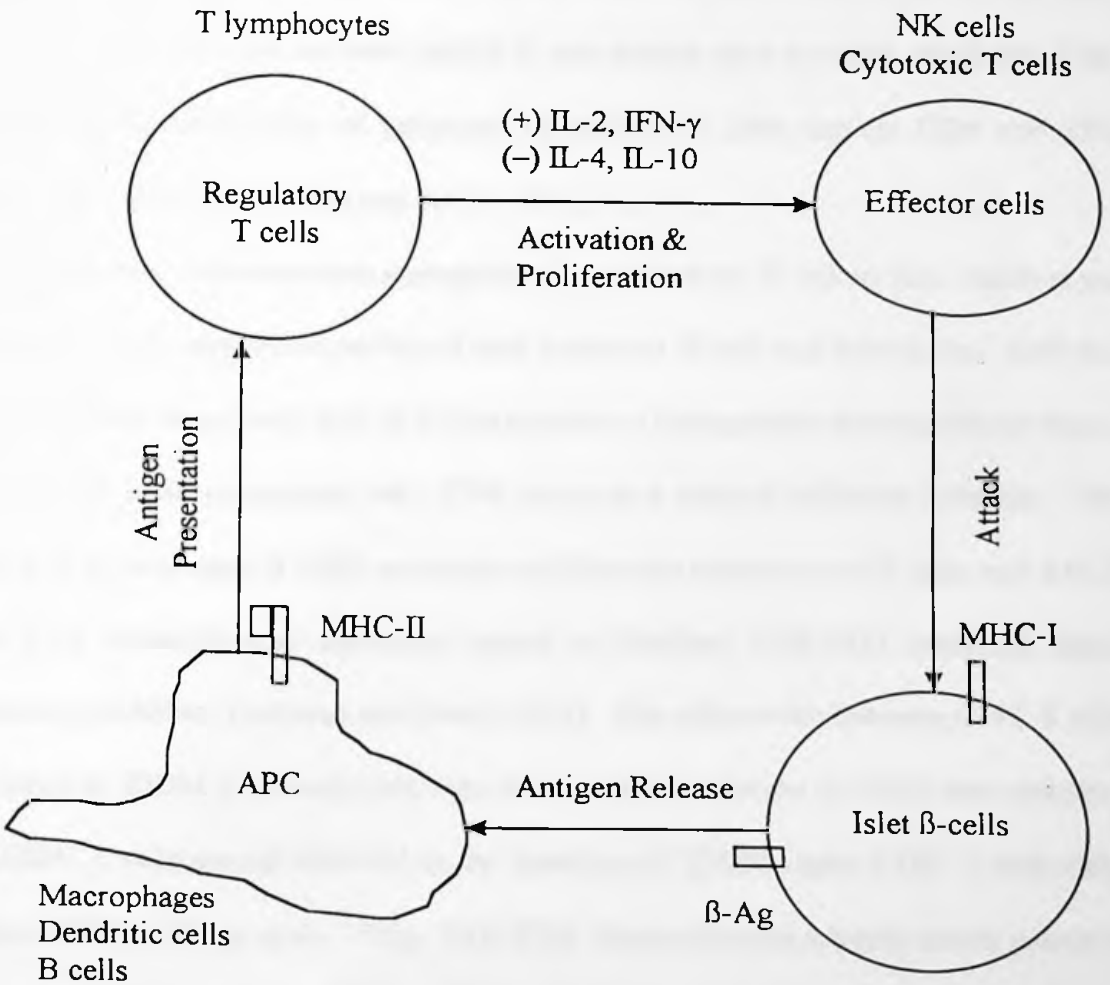
The immunomodulating effects of GT have been demonstrated *in vitro* (Waring, 1990; Waring, et al., 1988). GT has increased toxicity in irradiated mice, it delays the recovery of the immune system *in vivo* after immunosuppression by sublethal irradiation, and it induces apoptosis in the cells of lymphoid organs (Sutton, et al., 1994).

Immunology of Insulin Dependent Diabetes Mellitus

Pathogenesis

The immunologic events in diabetogenesis are summarized in Figure 3. The initial event in the immune response leading to IDDM is the uptake and processing of self-antigen by such antigen presenting cells (APC) as macrophages or dendritic cells. In association with surface MHC class I or class II molecules, APC present processed antigens to T helper (Th) cells. $CD4^+$ or $CD8^+$ Th cells with specific receptors recognize and bind to these class II or class I MHC and antigen complexes respectively. Th cell binding to the antigen-MHC complexes leads to Th cell activation and cytokine release. Cytokines, such as IL-2, activate toxic T cells, toxic macrophages, natural killers (NK) and other effectors, which contribute to the destruction of pancreatic islet ß-cells, resulting in IDDM (Rabinovitch,1993).

Figure 3. Induction of IDDM. This scheme presents an abbreviated model of possible immunologic interactions between pancreatic antigen presenting cells (macrophages, dendritic cells and B cells), regulatory lymphocytes (Th and Tc cells), effector cells (NK and Tc) and islet ß-cells leading to destruction of the pancreatic cells. In this model, β cell antigen is presented by APC in association with MHC II complex. This stimulates subsets of regulatory cells (helper T cells) which use cytokine secretion to activate effector cells (NK cells and cytotoxic T cells). The activated effectors attack insulin secreting pancreatic β cells. When most of the pancreatic β cells are destroyed, the host becomes diabetic.


T lymphocytes
NK cells
Cytotoxic T cells
(+) IL-2, IFN-γ
(–) IL-4, IL-10
Regulatory
T cells
Effector cells
Activation &
Proliferation
Antigen
Presentation
Attack
MHC-II
MHC-I
APC
Antigen Release
Islet ß-cells
ß-Ag
Macrophages
Dendritic cells
B cells

$CD4^+$ lymphocytes

CD4 and CD8 are T cell surface glycoproteins that are expressed on subsets of mature T cells with distinct patterns of MHC restriction. CD4 and CD8 serve as accessory molecules by facilitating interactions of T cells with APCs or cytotoxic T lymphocytes (CTL) with target cells. Both molecules are members of the Ig gene superfamily, but they are no more related to one another than to other members of the family. Approximately 65% of peripheral αβ-positive T cells express CD4 and 35% express CD8 (Abbas, Lichtman and Pober, 1991).

CD4 is a transmembrane glycoprotein, approximately 55 kD in size, which is expressed as a monomer on the surface of both peripheral T cells and thymocytes. CD4 has an extracellular region with four Ig V-like domains, a hydrophobic transmembrane region and a highly basic cytoplasmic tail. CD4 serves as a cell-cell adhesion molecule. The binding of CD4 to class II MHC molecules stabilizes the interaction of T cells with APCs. The CD4 molecule may transduce signals or facilitate TCR:CD3 mediated signal transduction (Abbas, Lichtman and Pober, 1991). The relationship between $CD4^+$ T cells and onset of IDDM is currently not very clear. An investigation of NOD mice indicated that $CD4^+$ T cells are not involved in the induction of IDDM, since $CD8^+$ T cells alone induced IDDM (Wong, et al., 1996). $CD4^+CD8^-$ thymocytes are a highly potent source of cells that have the ability to control autoimmune diabetes in rats. $CD45Rc_{low}$ $CD4^+$ T lymphocytes are considered as protective cells in IDDM prevention. Some investigators believe this cell subset to be synonymous with Th2 cells.

$CD8^+$ lymphocytes

Although CD8 is a transmembrane glycoprotein, the structure of the CD8 molecule varies among species and among T cells at different stages of maturity. It consists of either a homodimer of CD8α or a complex composed of CD8α and CD8β. Both CD8α and CD8β are members of the Ig superfamily with N-terminal extracellular Ig V-like domains, connecting peptides, a hydrophobic transmembrane region, and a highly basic cytoplasmic tail (Abbas, Lichtman and Pober, 1991). The relationship of $CD8^+$ T cells to IDDM and other autoimmune disorders has been discussed in a number of recent studies. Di-Cesare et al (1994) found that soluble CD8 levels were above the normal range in some newly diagnosed IDDM patients. Elevated levels of soluble CD8 at a time near diabetes onset may indicate that the activation of $CD8^+$ T cells is responsible for the autoimmune beta cell destruction. Using the method of depleting certain T cell subsets, Forwell and Mason (1993) demonstrated that the $CD8^+$ T cells were essential for the development of diabetes but not insulitis. Other investigators indicated that in development of diabetes in NOD mice, $CD8^+$ T cells function as an initiator to recruit $CD4^+$ T cells to islets as well as a final effector (Amano, et al, 1995). $CD8^+$ T cell subsets have been found important in other autoimmune diseases. Tada, et al. (1996) suggested that $CD8^+$ T cells play a role in the initiation and the recovery phase of collagen-induced arthritis (CIA). CIA incidence in CD8 -/- mice was significantly decreased compared with CD8 +/- mice. Interestingly, $CD8^-$ deficient mice were more susceptible to a second induction of arthritis after remission of initial disease, pointing towards an immunoregulatory role for $CD8^+$ T cells.

$RT6^+$ lymphocytes

As shown in Table 4, RT6 is a T cell alloantigen that occurs in at least two forms, RT6.1 and RT6.2. RT6 develops extrathymically and is expressed on about 50% of T helper cells and on about 60% of cytotoxic T cells (Jiang, et al., 1990). Depletion of $RT6^+$ T cells in DR/BB rats can induce diabetes while transfusion of $RT6^+$ T cells from DR/BB rats into DP/BB rats prevents diabetes, proving that $RT6^+$ T cells play a critical role in the pathogenesis of BB rat autoimmunity, perhaps by regulating autoimmune T cells (Woda et al., 1991; Whalen, 1994).

RT6 is one component of a multi-subunit receptor complex. RT6 is associated with *src* family tyrosine kinases and generates an activation signal (Rigby, et al., 1996). In this model, RT6 interacts with at least five cytoplasmic proteins, including $p56^{lck}$ and $p60^{lyn}$, mediated by an as yet unidentified linker molecule analogous to gp130. PKC activation is able to modulate RT6 signaling. RT6 delivers an "accessory" signal that increases expression of cytokine receptors and enhances the ability of these T-cells to enter the cell cycle in response to growth factors like IL-2 and/or IL-4. The activation signaling pathway involving RT6 could provide a control point for activation of an antigen-engaged regulatory T-cell population, thereby modulating immune function (Table 4).

Natural killer cells

Natural killer (NK) cells are a subset of lymphocytes with prominent cytoplasmic

Table 4. Characteristics of the RT6 rat T cell alloantigen (Crisa, et al., 1992)

Marks 60-70% of peripheral T cells
Two genes, designated $RT6^a$ and $RT6^b$, encode two surface proteins, RT6.1 and RT6.2, respectively
RT6 is linked to the cell surface by a phosphatidylinositol anchor
RT6.1 consists of a 24-26 kDa nonglycosylated peptide plus at least five additional differentially glycosylated polypeptides of 30-35 kDa
RT6.2 corresponds to a 24-26 kDa nonglycosylated polypeptide
RT6 maps to linkage group I on rat chromosome 1
RT6 is not expressed on thymic or bone marrow cells
>1/2 of $CD4^+$ T cells are $RT6^+$
>2/3 of $CD8^+$ T cells are $RT6^+$
>1/3 of $CD4^+$ $CD45R^+$ T cells are also $RT6^+$ (in the DR-BB rat)

granules and are found in blood and lymphoid tissues, especially the spleen. NK cells are viewed as CTLs that lack the specific T cell receptor (TCR) for antigen recognition. Although the target specificity of NK cells is broader than that of CTLs, it is not random. NK cells express CD2 and CD16. As with CTLs, NK granule exocytosis and secretion of a cell toxin are involved in killing by NK cells. NK cells may be involved in some autoimmune processes, such as graft-versus-host disease (GVHD) among bone marrow transplant recipients (Abbas, Lichtman and Pober, 1991). For years, functions of NK cells in IDDM pathogenesis have been discussed. Jiang et al (1994) found a relative abundance of Tc and NK cells in the islets of acutely diabetic DP and RT6-depleted DR rats. They also found the expression of cytolysin mRNA which encodes a cytolytic pore-forming protein produced by both Tc and NK cells. They demonstrated that in the islets of acutely diabetic DP rats and RT6-depleted DR rats, NK cells were more abundant than in normal rats. Contrary results have been obtained in another study. Shachner et al (1992) argued that CTL cells played a role in pathogenesis of the disease and NK cells are not necessary for autoimmune islet destruction.

Th1 and Th2 lymphocytes

Th1 and Th2 cell subsets are generally $CD4^+$, but may be $CD8^+$. Antigen-activated Th cells aid in mediating both cellular and humoral (antibody) immune responses. At least two distinct Th-cell types, Th1 and Th2, have been described in humans, mice and rats based on their distinct cytokine secretion patterns. Th1 cells produce IL-2, IFNγ, and TNF-ß (lymphotoxin), whereas Th2 cells produce IL-4, IL-5, and IL-10. Other cytokines,

such as IL-3, GM-CSF and TNFα, are produced by both Th1 and Th2 cell populations. Th1 and Th2 cell subsets, releasing distinct patterns of cytokines, lead to different T cell actions (Rabinovitch, 1993; Paul and Seder, 1994).

Th1 cells and their cytokine products mediate cellular immunity (delayed-type hypersensitivity). IFNγ and TNFß activate vascular endothelial cells to recruit circulating leukocytes and activate cytotoxic macrophages to eliminate the antigen-bearing cells. IL-2 and IFNγ activate CTLs to kill target cells with MHC-associated antigen and activate NK cells to kill target cells in an MHC-independent fashion. The production of cytokines by Th1 and Th2 cells are mutually inhibitory which creates a situation of dynamic balance between these two cell classes. IFNγ inhibits the production of Th2 cytokines while IL-4 and IL-10 inhibit production of Th1 cytokines. Cytokines secreted by Th1 cells will activate effector cells, including cytotoxic macrophages, cytotoxic T cells and NK cells, and will be expected to promote IDDM. Meanwhile, cytokines of Th2 cells will inhibit those effector cells and actually protect from autoimmune disease.

Immune regulation through apoptosis

Regulation of immune function occurs by various mechanisms including deletion of certain cell types. The life and death of lymphocytes is tightly controlled by membrane receptors that activate either proliferative or apoptotic (programmed cell death) processes. Fas (also known as Apo1 or CD95) antigen, a 48 kDa surface protein, has been identified as a key receptor involved in apoptotic death among lymphoid cells. Mutations in the gene which encodes Fas are responsible for lymphoproliferative disorders and an

associated lupus-like syndrome in lpr and lpr^{CR} mice. Defects in Fas-induced apoptosis may lead to incomplete elimination of peripheral autoreactive cells in these mice. A recent investigation found that patients with lymphoproliferative syndromes and autoimmune disorders have a large deletion in the Fas gene and no detectable Fas expression (Rieux-Laucat, et al., 1995).

Autoreactive T cells die by means of apoptosis during thymic development by a negative selection process (Ogasawara, Suda and Nagata, 1995). In addition, autoreactive mature T cells are also deleted in the periphery. Fas is expressed in activated mature T cells, and Fas-mediated apoptosis occurs during the induction of peripheral tolerance and/or in the antigen-stimulated suicide of mature T cells (Nagata, 1994). These findings illustrate the crucial role of Fas in apoptosis and may provide a molecular basis for some autoimmune diseases in humans.

IDDM Prevention by Immune Manipulation

Diabetes prevention

In recent decades, many therapeutic intervention strategies have been proposed and studied for IDDM prevention. Most have involved experimental animals, but some have been applied to human subjects. Several methods, summarized in Table 5, are known to reduce IDDM in animals (Bowman, Leiter and Atkinson, 1994). Immunosuppressive drugs, cyclosporin A (CsA) and FK-506, can effectively prevent or delay IDDM in BB rats and NOD mice (Mori, et al., 1986; Miyagawa, et al., 1990). Treatments that compromise the effector functions or viability of either $CD4^+$ or $CD8^+$ T cell subsets can

Table 5: Therapies that prevent diabetes in NOD mice
(Bowman, Leiter and Atkinson, 1994)

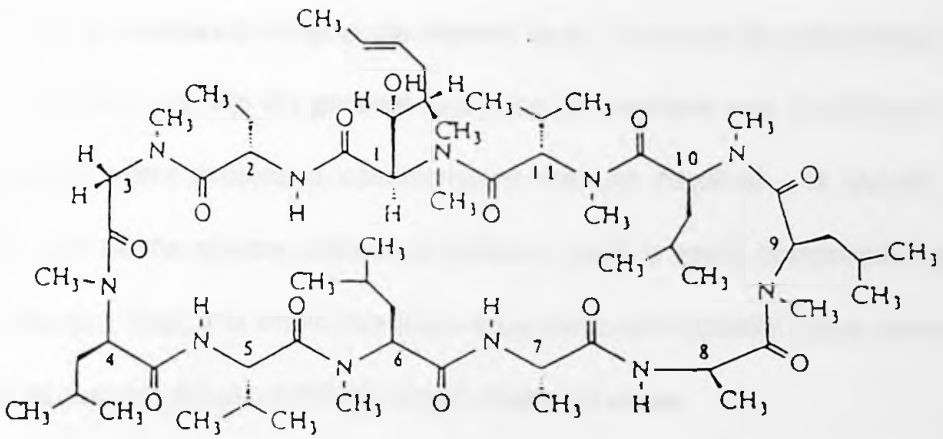
Immunosuppression	T-cell functions:	neonatal thymectomy anti-lymphocyte serum anti-Thy-1 anti-CD3 anti CD4 anti CD8 cyclosporin FK-506
	Macrophage/APC (antigen presenting cell) function:	anti-complement receptor silica LDHV (Lactate dehydrogenase virus) anti-MHC (major histocompatibility complex) class I anti-MHC class II blocking peptide for anti-MHC class II anti-IFN-γ (interferon γ)
Immunostimulation	Pathogenic viruses:	LCMV (lymphocytic choriomeningitis virus) EMCV (encephalomyocarditis virus) MHV (murine hepatitis virus)
	cytokines or cytokine inducers:	IL-1 (interleukin-1) TNF-α (tumor necrosis factor α) IL-4 IFN-γ poly [I:C] ConA (concanavalin A)
	Others:	CFA (complete Freund's adjuvant) BCG (Bacille Calmette Guerin) OK432 heat-shock protein 65
Tolerance induction		bone-marrow transplantation intrathymic islet transplantation oral insulin dendritic cells from pancreatic node neonatal tolbutamide treatment immunization with insulin or insulin B chain
Manipulation of hormonal/dietary milieu		gonadectomy prophylactic insulin treatment diazoxide elevated temperature semi-purified diets
Anti-inflammatory agents		Nicotinamide superoxide dismutase-desferrioxamine vitamin E aminoguanidine

retard or circumvent diabetes (Maki, et al., 1992; Sempe, et al., 1991). Meanwhile, immunostimulation is also used in IDDM prevention. Some viruses prevent IDDM in experimental animals by means of stimulating cytokine production and other immune responses. Therefore, it is not surprising that cytokine treatment and cytokine induction are also useful methods in those animal studies. Therapeutic protocols, such as oral insulin administration (Atkinson, Maclaren and Luchetta, 1990), insulin vaccine to induce tolerance (Williams, et al., 1993), and the injection of dendritic cells or splenocytes (Clare-Salzler, et al., 1992), may induce regulatory tolerance or clonal anergy in T lymphocytes reactive to islet antigens. Recent studies indicated that intrathymic or intravenous administration of recombinant GAD-65 results in acquisition of T cell tolerance to β-cell autoantigen (Kaufman, et al 1993; Tisch, et al., 1993). Hormonal or dietary manipulation, and anti-inflammatory agents are also reported as effective therapeutic interventions for IDDM in BB rats and NOD mice. Because GT is an immunomodulator and immunosuppressive factor, it may also have a role in IDDM prevention, as will be elaborated later.

Immune modulators

Drugs regulating apoptosis would also be anticipated to have therapeutic or preventive value in autoimmune diseases if the induced apoptosis involved an appropriate class of cells such as autoreactive lymphocytes or regulatory cells that would otherwise promote cytotoxic reactions against pancreatic cells. CsA (Figure 4) and its analogue, FK506 (marketed under the name, Tacrolinus or Prograff), are used in transplantation medicine to increase survival rates of kidney and other organ transplantations (Borel,

Figure 4. Structure of cyclosporin A (CsA). This immune suppressive compound is used clinically in transplantation medicine. The compound is a macrocyclic fungal product and in comparison to GT (see Figure 1) shows a vastly different chemical structure. It has no bridge moieties as GT does. CsA has been used to block autoimmunity in experimental animal models, although it displays significant renal toxicity which limits its long term use.



Cyclosporin A

1990). CsA and FK506 inhibit T cell response to the allograft (Thomson, 1992), by inhibiting the expression of lymphokine genes, including IL-2, IL-3, IL-4, GM-CSF, TNFα, and IFNγ. Cytokine inhibition results from interference with the synthesis or transport of several nuclear proteins into the nucleus, or is due to inhibition of their functional activation (Schreiber and Crabtree, 1992). CsA blocks apoptosis by inhibiting the DNA binding activity of transcription factors (Erlanger, 1992; Shi, Sahai and Green, 1989). Because of its immunosuppressive effects, CsA has also been proposed for use in IDDM and other autoimmune diseases. In a sense, the promotion of allograft survival may be conceptually parallel to pancreas survival in the diabetic host. However, the side effects of CsA, primarily nephrotoxicity, are the greatest limitation to extensive use, particularly in autoimmune diseases where prolonged administration may be required. It should be remembered that one of the adverse effects of diabetes itself is renal compromise and ultimately renal failure. Thus, it is ironic that CsA treatment could actually cause some of the same symptoms that the disease (IDDM) would otherwise cause.

Cyclosporin A, FK506, reticuloendothelial blockade, anti-NK cell antibody, and other treatments have been tried in DP/BB rats. These treatments are not clinically satisfactory because of significant toxicity or other side effects. These immunologic reagents are able to prevent diabetes in DP/BB rats, indicating that the autoimmune reaction may be interdicted at several different points, including the generation or activation of autoreactive lymphocytes, the restoration or activation of regulatory cells, the inhibition of effector cells which participate in destruction of the pancreatic ß-cells, and unregulated suppression or release of cytokines. These points of autoimmune

interdiction provide a conceptual approach with which to study the potential beneficial effects of GT in diabetogenesis among disease prone rats.

A possible role for gliotoxin in autoimmunity and diabetes

As noted above, IDDM is a complex autoimmune disease that may ultimately be treatable by immunologic interventions that alter the function of autoreactive lymphocytes, augment the function of protective regulatory cells or prevent the toxic action of such effector cells as macrophages and NK cells that destroy pancreatic ß-cells (Figure 3). CsA has value as an immunosuppressive therapy, but some severe side effects limit its clinical use. GT is very different chemically from CsA and may be exploited either therapeutically or as an immunologic reagent to prevent or treat type I diabetes and other autoimmune diseases. The work described here will provide a better understanding of the mechanism involved in diabetes prevention by GT and reveal whether GT or one of its congeners has value as a potential new drug candidate to prevent or treat diabetes.

Diabetes prevention by GT may result from its action on one or more of several cell types, including APC (macrophages or dendritic cells), regulatory Th cells, effector Tc or NK cells, or target islet ß-cells. If GT affects regulatory T cells, it may permit restoration of certain subtypes of T cells, such as the critical $RT6^+$ T cells, since DP/BB rats are known to have a low number of $RT6^+$ T cells. GT might also interfere with cytokine production to change either cytokine types or quantity, block or enhance cytokine-mediated effects or alter the balance of cells, dependent on the dynamics of cytokine production. Since the DP/BB rat has an increased number of NK cells and few

$CD8^+$ Tc cells, if GT acts on effector cells, it may reduce the number or/and activity of NK cells.

Hypothesis

The hypothesis for this study is that GT treatment prevents IDDM in DP/BB rats. GT treatment would be expected to exert its effects on the immune system by elimination of antigen presenting cells, or cytotoxic effectors or by altering the balance or function of regulatory cells. Because apoptosis is known to be the mechanism for protection against dangerous immunologic reactions, GT may act by inducing apoptosis of some types of leukocytes. GT may alter immunologic reactions by restoring other cells especially regulatory T lymphocytes.

GT functions as an immunomodulator with immunosuppressive effects as revealed by research completed in the past decade, and has been used to suppress immune responses in transplantation. We reasoned that GT's immunosuppressive function should help to block the autoimmune process and prevent insulin dependent diabetes and possibly other autoimmune diseases. GT treatment may be able to decrease the incidence of IDDM or perhaps delay its onset in DP/BB rats.

Regulatory T lymphocytes, such as $CD4^+$ T cells, $CD8^+$ T cells and $RT6^+$ T cells, play a key role in IDDM development and it is appropriate to discover whether GT can exert an effect on these cells or whether its effects are concentrated in the antigen-presenting or late effector stages of autoimmunity. If regulatory T lymphocytes are targetted by GT, the autoimmune process as well as IDDM in DP/BB rats may be

prevented in a most advantageous manner since alteration of antigen presentation or immune effector function may have a harmful effect on cancer surveillance and antimicrobial immunity.

Apoptosis is considered an important mechanism by which the immune system eliminates dangerous cells and limits immune responses. Apoptosis should help inhibit autoimmunity and reduce untoward immune responses in the host. Since one study (Sutton et al. 1994) had reported that GT induced apoptosis *in vitro*, we proposed that GT might also induce apoptosis *in vivo* and inhibit the autoimmune processes of IDDM, although *in vivo* apoptosis induction by GT has never previously been demonstrated.

Significance of this research

The most important potential contributions of this project are 1) gaining new insight into the way in which GT interferes with the development of autoimmunity with IDDM as a model; 2) providing preliminary evidence that a new category of chemopreventive agents may provide improved quality of life for those predisposed to development of IDDM; and 3) having a better understanding of IDDM pathogenesis.

MATERIALS AND METHODS

Experimental Animals

Equal number of male and female diabetes prone BioBreeding (DP/BB) rats were purchased from the National Institues of Health central breeding colony at the University of Massachusetts Medical School (Worcester, MA). DP/BB rats were shipped in filter crates and were maintained in isolation from other animals and humans (viral antibody free conditions). The vivarium performs periodic serologic testing on sentinal animals to insure that specific pathogens have not entered the facility. Upon arrival, animals were caged individually and given one week to become acclimated to the animal facility. The environment was temperature and light (12 hours light/12 hours dark) controled. Animals were provided food and water ad libitum. Water bottles were sterilize before reuse, but food, water and bedding were not. Standards for animal care of BB rats have been published by Olsen et al (1990). Investigators and animal caretakers donned mask, cap, gloves and gown before entering the animal rooms to avoid. All materials for these rats were treated to minimize contamination.

Toxicity Study

RIN 38 cell cytotoxicity and function

The RINr 1046-38 (RIN 38) cell line was originally derived from an insulinoma. This cell line was maintained in the laboratory of Dr. Bruce Chertow. To estimate the highest concentration of GT which is non-toxic to β-cells, RIN 38 cells were grown 48

hours in RPMI-1640 cell culture medium with 5% FBS and 2.8mM glucose at 37°C in a CO_2 (5%) incubator. At the end of 48 hours, cells were washed with Krebs-Ringer buffer (KRB). To 1 ml of KRB, glucose (2.8 mM) was added to each well for a 1 hr pre-incubation at 37°C in a CO_2 (5%) incubator. This medium was aspirated, cells were gently washed with KRB, and 1 ml KRB containing 2.8 or 16.7 mM glucose and gliotoxin (0, 0.5,1, or 5 mg/ml) was added. Cells were incubated 1 hr at 37°C in a CO_2 (5%) incubator. Supernatant was removed after 500 x g centrifugation at 4°C. The supernatant was frozen for RIA assay. The viable RIN 38 cell counts were determined by trypan blue staining and counted with a hemacytometer. Viable cells were counted as those that excluded the blue stain.

The RIN 38 cells are insulin secreting and were used to assess effects of GT on insulin secretion. To estimate the effect of GT on insulin secretion, RIA assay for insulin was conducted in Dr. Chertow's laboratory using the frozen supernatant mentioned above.

To iodinate insulin, 10 µl insulin (0.4µg/µl), 20 µl 0.25 M phosphate buffer (pH7.5), 14.2 mCi ^{125}I and 15 µl chloramine were combined. After 45 seconds, 50 µl metabisulfate and 25 µl 12.5% BSA were added in phosphate buffer. This mixture was removed by passage through a carboxymethyl cellulose column in a syringe and washed with 0.05 M phosphate buffer. The sample was washed with 2.5% BSA and 0.5 ml collected into each of six tubes. The tubes were counted by gamma counter and fractions in the last two tubes were used as radiolabeled insulin.

Guinea pig anti-porcine insulin, which crossreacts with rat insulin was used at 1/500,000 dilution. A standard curve consisting of 0, 0.21, 0.58, 1.33, 2.83, 5.63, 11.25 ng insulin per ml was constructed. The samples were combined with the antibody for four

hours at room temperature. Radiolabeled insulin was then added to each tube. After overnight incubation, 2 ml of dextran-coated charcoal (25 mg/ml) was added to each tube to remove unbound insulin and centrifuged for 20 minutes at 500 x g at 8 °C. The supernatant was counted on a gamma counter set for ^{125}I for 12 minutes per tube. Radioactivity of each unknown sample was compared to the standard curve to determine insulin concentration. All samples were assayed in duplicate and results reported as mean.

Peripheral leukocyte counts

After receiving 10 doses of GT or vehicle, blood was collected from GT treated and control rats preparing the rat's tail with 70% isoproposol by snipping the tip of the tail with alcohol-treated scissors. A drop of blood was allowed to form and 20 µl of blood was drawn into a Unopette capillary (Becton-Dickinson, Rutherford, NJ). Blood specimens were diluted to 2 ml in buffered ammonium oxalate solution. After red cell lysis, leukocyte count was performed microscopically with a hemacytometer and expressed asleukocytes per milliliter.

Histology and Morphometry

Liver, kidney, spleen and pancreas tissues were removed from GT treated DP/BB rats (diabetic and pre-diabetic) and untreated control DP/BB rats and placed in 10% formaldehyde. The tissue samples were embedded in paraffin and 10 micron sections were cut.

Routine hematoxylin and eosin (H&E) staining were applied to make slides for histological observations. The sections were covered with permount and coverslips. The slides were stored in flat slide holders until permount dried.

For mophometric evaluation of splenic follicles a video camera with television monitor was placed on the microscope and images of splenic follicles placed in the center of the monitor's viewing area. A vernier caliph was used to make measurements directly from the monitor. All evaluations were done at the same magnification so results from different tissues would be comparable.

Research Design (Immunology Studies)

To evaluate the effect of GT on relevant cells of the immune system, both direct effects of GT on isolated spleen cells (*in vitro* study) and the effects resulting from chronic animal treatment (*in vivo* study) were investigated. For these studies only non-diabetic (pre-diabetic) animals were used. By harvesting cells at 65 days of age, we were assured that all cells were recovered from pre-diabetic animals.

In vitro study

For the *in vitro* investigation of GT effects on splenic lymphocytes, untreated DP/BB rats were sacrificed at 65 days of age and splenic cells were isolated as described below. Half of the splenic cells were incubated for one hour with 1 μg/ml GT at 37°C in a CO_2 (5%) incubator while the other half was cultured in GT-free cell culture medium as the control. The suspending vehicle, ethanol, was added to each control sample in an amount equal to that present in GT treated samples. Both GT treated samples and

controls were divided into two aliquots. One aliquot received 10 μg/ml concanavalin A (ConA) stimulation and another remained unstimulated, yielding four different preparations of splenic cells: (1) GT treated cells; (2) ConA stimulated GT treated cells; (3) controls; and (4) ConA stimulated controls. These cells were then labeled with mAb for lymphocyte surface markers, analyzed by TUNEL method for lymphocyte apoptosis, or used in proliferation tests, each described below.

***In vivo* study**

For *in vivo* study of lymphocytes from GT-treated rats, experimental animals were placed into two groups. Half of these DP/BB rats were treated with 1 μg/g body weight GT three times weekly (Monday, Wednesday and Friday) from 30 days of age and continuing until 65 days of age. The remaining animals received the same volume of suspending vehicle (glycerin) according to a schedule identical to that were for GT treated rats. All rats were monitored for weight gain to verify that they were not becoming diabetic and were sacrificed at 65 days of age. Spllens and pancreases were recoverd and used as described below.

Splenocytes from GT treated rats and control rats were divided into two aliquots. One aliquot was stimulated with 10 μg/ml ConA and the remaining cells were kept unstimulating. Four different samples were prepared: (1) splenocytes from GT treated rats; (2) splenocytes from GT treated rats with ConA stimulation; (3) splenocytes from control rats; (4) splenocytes from control rats with ConA stimulation. Samples were then used for surface marker labeling, apoptosis analysis and proliferation testing as described subsequently.

In Vivo Gliotoxin Use

Treatment regimen

GT was obtained from Sigma Chemical Company (St. Louis, MO) and suspended in sterile glycerol at a concentration of 1 mg/ml. Control group rats received suspending vehicle only. GT-treated animals received 1 μg/g of body weight three times weekly (Monday, Wednesday and Friday). All injection were administered intraperitoneally and volumes injected ranged from 0.1 to 0.25 ml depending on the weight of the animal. Animals were monitored for development of diabetes throught the duration of their treatment..

Diabetogenesis

The onset of diabetes was heralded by a decline in weight, positive urine glucose test and confirmed by elevated blood glucose (>200 mg/dl). As soon as diabetes was confirmed, the diabetic animals were painlessly euthanized by nembutal overdose and exsanguination via cardiac puncture. Prior to each GT or control injection the animal was weighed, the weight compared to the previous weighing and if there was a decline in weight, urine was tested with a glucose test paper. If glycosuria was present, a small drop of blood was obtained by snipping the tail with sterile scissors and the blood glucose was measured with a glucose meter. The animal was considered diabetic if blood glucose was greater than 200 mg/dl.

Serum glucose measurement

Serum glucose was measured by the method of modified Keston's procedure (Raabo and Terkildsen, 1960). Blood from an experimental animal was allowed to clot on ice. The clot was released from the sides of the tube with a wooden splint and blood was centrifuged in the cold for 15 minutes at 900 x g. Serum was removed with a Pasteur pipet and frozon at -20°C until analyzed.

Reagents for this test were obtained from Sigma Chemical Company (St.Louis, MO). Water (2 ml) was added to a test tube as a blank control. Water (1.8 ml) and 0.2 ml glucose standard solution (100 mg/dl) was added to another test tube as a standard. Serum samples were prepared by precipitating of protein with Barium sulfate. Serum was diluted 1:10 with distilled water to 2 ml. Diluted serum and mixed with 1 ml of 0.3 N zinc sulfate. Barium hydroxide (1ml, 0.3 N) was added. After mixing well, 0.5 ml of deproteinized serum supernatant was transfered to another tube and 5.0 ml combined PGO enzymes (5 units/ml glucose oxidase and 1 units/ml peroxidase)-color reagent solution (o-Dianisidine dihydrochloride 2.5mg/ml) was added. After mixing, tubes were incubated at room temperature (18-25 °C) for 45 min. At the end of the incubation period, absorbance of samples was determined spectrophotometrically at 450 nm read. Serum glucose values were calculated by the following equation:

Serum Glucose (mg/dl) = [Absorbance of Test / Absorbance of standard] x 100

Immunologic Effects of Gliotoxin

Isolation of splenic leukocytes

As noted previously, *in vitro* lymphocytes studies employed cells from rats 65 days of age. The *in vivo* GT treatment study employed splenic cells from rats treated with GT or control injections during the period from age 30 days to 65 days. At 65 days experimental animals were euthanized by CO_2. A midline incision was mad expose the spleen which was aseptically removed and placed in 10 ml of Dulbecco's phosphate buffered saline (PBS) on ice for transport from the vibarium to laboratory. In the laboratory, splenic tissue was minced and further disrupted by passage through a sterile 80 mesh tissue sieve using aseptic technique in a laminar-flow hood. Cells were dispersed by repeated aspiration and expression with a sterile 21 gauge needle, washed twice with 15 ml of Dulbecco's PBS, and recovered by centrifugation at 1000 rpm for 4 min. Isolated leukocytes were suspended in Eagle's Minimum Essential Medium (MEM) with 10% FCS, and unless otherwise noted, incubated in a 25 ml tissue culture flask in CO_2 (5%) at 37°C to remove adherent cells. Viable cell counts of non-adherent cells were determined by 0.4% trypan blue staining and counting in a hemacytometer. All lymphocyte preparations used in this study showed >95% viability.

Lymphocyte surface marker labeling

Splenic lymphocyte subsets were stained with antibodies against surface CD4, CD8, NK, RT6, PanT and MHC II prior to flow cytometric analysis. Reagents used include mouse anti-rat CD4 (clone OX-35), CD8 (OX-8), PanT (OX-19) monoclonal antibodies (mAb) which were purchased from Pharmingen, San Diego, CA. Mouse anti

rat NK (3.2.3) mAb was purchased from Harlan Bioproducts, Indianapolis, IN. Mouse anti rat RT6 (P4/6) and MHC II (OX-17) mAb were purchased from Biosource, Camarillo, CA. With the exception of RT6, all antibodies were flurescent labled by the supplier. Since anti RT6 mAb is unlabeled, a goat anti-mouse secondary antibody was used to visualize the anti-RT6 antibody. The fluoresceint isothiocyanate (FITC)-labeled secondary antibody was purchased from Sigma, San Louis, MO. FITC labeled anti CD8 mAb was paired with phycoerythrin (PE) labeled anti CD4 mAb for double staining. FITC labeled NK mAb was paired with PE labeled PanT mAb for double staining.

To control for nonspecific staining by antibodies, FITC labeled mouse IgG1 and PE labeled mouse IgG2a isotype control antibodies were purchased from Sigma, St. Louis, MO and used for each lymphocyte sample mentioned above.

Prior to analysis of lymphocyte surface markers, each antibody was titrated to determine an ap propriate antibody dilution. Splenocytes from normal rats were used for titration. Anti CD4 and CD8 mAbs were diluted 100 fold in PBS while other mAbs were diluted 50 fold. Splenic lymphocytes were harvested by centrifugation and resuspended to a concentration of 10^6 cells/ml in Dulbecco's PBS with 1% FBS. Splenic lymphocytes were added to a series of tubes (10^5 cells/tube) and incubated with equal volumes of FITC and PE labeled mAbs at appropriate dilutions for 30 minutes on ice. Stained cells were washed three times with assay buffer (1 mg/ml sodium azide in PBS). The secondary antibody was applied to unlabeled anti RT6 mAb after primary antibody binding. The labeled cells were fixed by adding 500 µl of 4% paraformaldehyde. The fixed cells were held at 4°C overnight for flow cytometry evaluation the next day.

Flow cytometry

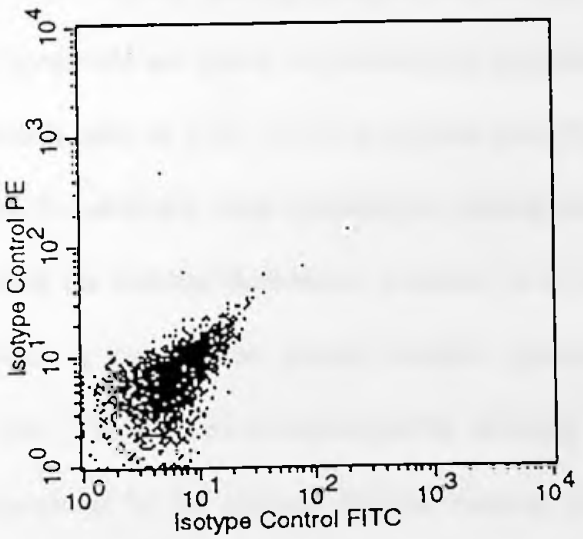
Flow cytometric analysis was performed in a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, CA). The argon ion laser was operated at 488 nm with 15 mW of power. Fluorescence was determined using logarithmic amplification. Forward scatter (FSC) and side scatter (SSC) were determined using linear amplification. A MacIntosh computer with Cell Quest® software was used to record, convert and analyze data.

For each sample, the flow cytometer scanned at least 10,000 cells and recorded four parameters: FSC, SSC, FITC staining (FL1) and PE staining (FL2). The lymphocyte populations were gated from FSC and SSC plots and were replotted into a dot plot or a histogram plot. The data derived from dot plot included the percentage of FITC or PE labeled cells in total gated lymphocytes.

Evaluation of Fluorescent Intensity

The isotype control tests were conducted using FITC conjugated mouse IgG1 and PE conjugated mouse IgG2α antibody to determine any nonspecific staining as shown in Figure 5. For analysis of $CD4^+$, $CD8^+$, $CD5^+$ (PanT) MHC II^+ and NK lymphocytes, we first recognized and gated the lymphocyte populations on the FSC versus SSC dot plots. The gated data were replotted as FITC (FL1) versus PE (FL2) dot plots. Quadrants were drawn on the basis of stained versus unstained cells as determined by isotype control data on regative control data to permit calculation the percent of cells in each quadrant.

Figure 5. Example of isotype control data for primary antibodies. Isotype controls were evaluated for each lymphocyte sample analyzed to establish the level of non-specific staining expected from each of the primary antibodies (anti CD4, anti CD8, anti NK, anti CD5, anti MHC II). The primary antibodies for CD8, MHC II and RT6 are mouse IgG2α and others are mouse IgG. Therefore the isotype control antibodies were FITC-conjugated anti mouse IgG2α mAb and PE conjugated anti mouse IgG mAb. After staining cells with isotype control antibodies, the lymphocytes were gated based on the scatter plot (not shown) of cells in this preparation, and were replotted in the FL1-FL2 plot, of which this figure is an example. Since no specific staining of these cells was possible, all cells in the FL1-FL2 plot are considered to be negative. Positive cells will therefore be found to the right of the FL1 marker and above the FL2 quadrant markers.


Isotype Control PE
Isotype Control FITC
10^0
10^1
10^2
10^3
10^4

Quadrants therefore represented FITC stained, PE unstained cells; FITC unstained, PE stained cells; double stained and double negative cells (Figure 6).

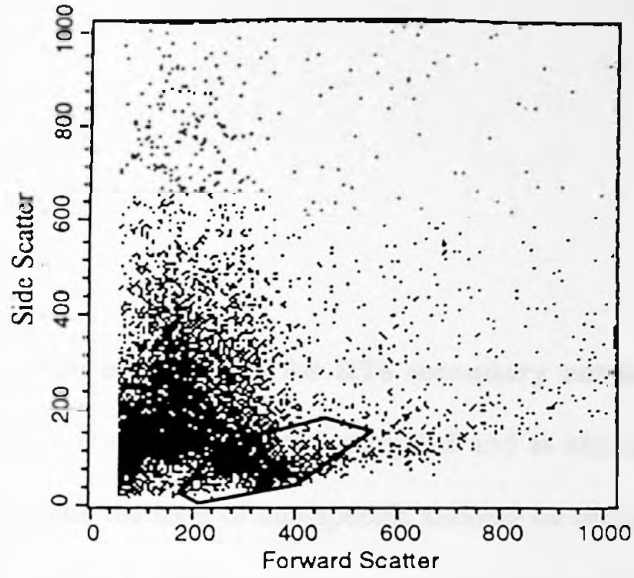
Primary mouse anti-rat RT6 mAb is unlabeled so that an FITC conjugated secondary goat anti mouse IgG mAb is required to visualize the RT6 staining. A secondary antibody negative control was conducted to determine any nonspecific staining as shown in Figure 7. The $RT6^+$ lymphocyte data were first analyzed as $CD4^+$, $CD8^+$, $CD5^+$ (PanT) and NK lymphocytes. Since DP/BB rats did not have a distinctly staining $RT6^+$ lymphocyte population, a count of $RT6^+$ staining lymphocytes was not possible. For analyses of $RT6^+$ cell data, we recognized and gated the lymphocyte population from FSC versus SSC dot plots then replotted them to FITC (FL1) histogram plots (Figure 8). The intensities of fluorescent staining for each dot were recorded as channel number and the mean channel number representing the average fluorescent intensity of all cells with any RT6 surface stain was calculated. A higher mean channel number represented a larger number of RT6 molecules per cell. The data were normalized by dividing mean channel numbers for experimental preparations by the average channel number for all samples analyzed during a given flow cytometry section to produce a mean channel index.

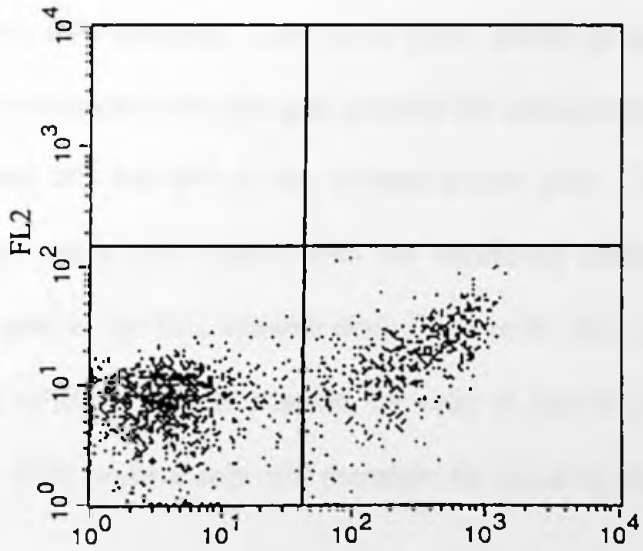
Pancreatic immunohistology

Pancreatic tissue was removed from GT treated DP/BB rats and untreated control rats, placed in 1 ml centrifuge tubes and immediately immersed in liquid nitrogen and then moved to a -80°C freezer where they remained until sectioning.

Pancreatic tissue was embedded in Tissue Freezing Medium (Sakura Finetek, Inc., Torrance, CA) and sections were cut with a Cryocut 1800 cryostat (Reichert-Jung,

Figure 6. Example of method used to identify % of cells positive for a given surface marker. To evaluate the percentage of spleen lymphocytes bearing a particular surface marker (MHC II shown here), rat splenocytes were incubated with fluorescent conjugated antibodies (FITC-conjugated mouse anti rat MHC II mAb). The upper panel shows the scatter plot of cells in this preparation with the gate selected for lymphocytes indicated by the polygon shown between 200 and 600 on the forward scatter axis. The lower plot indicates the staining seen among gated cells treated with the FITC-conjugated mouse anti rat MHC II mAb. The antibody staining will only be seen in the FL1 channel and there is no FL2 signal present. The right lower quadrant shows cells positive for FITC staining. The cells in left-lower quadrant are considered to be negative. The table represents the computerized tally of cells in each quadrant.



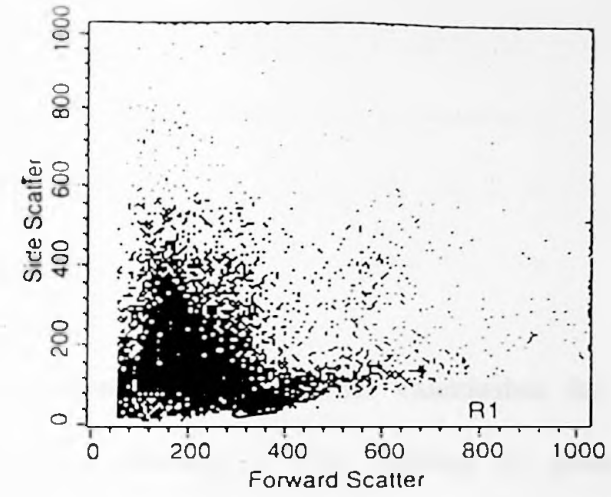


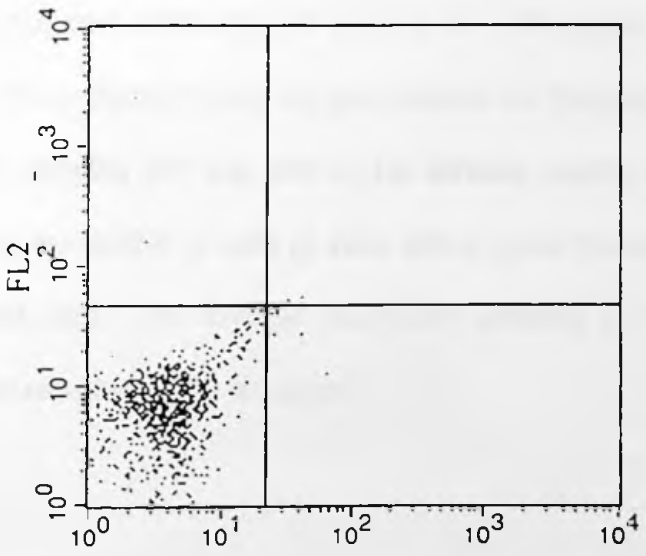
FL1: FITC labeled MHC II mAb staining
for GT untreated control sample V3

Total events: 10,000
Gated events: 1,761
Quadrant location: 41,146

Quad	Events	% Gated	% Total
UL	0	0.00	0.00
UR	0	0.00	0.00
LL	1137	64.57	11.37
LR	624	35.43	6.24

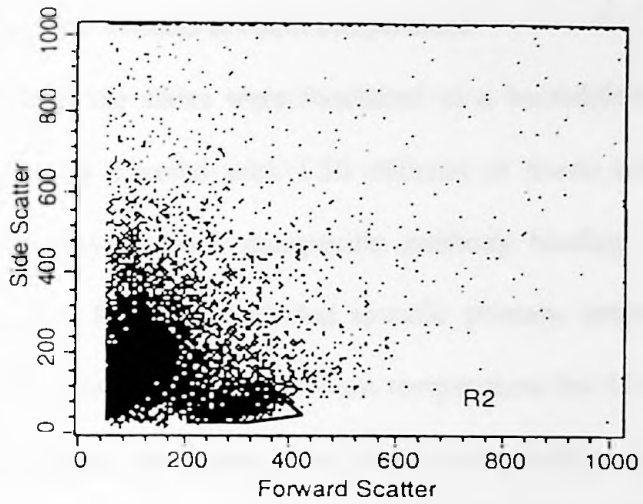
Figure 7. Example of isotype control data for RT6 secondary antibody. For RT6 staining, the primary antibody is not fluorescent conjugated and is identified by labeled secondary antibody. To evaluate the level of non-specific staining by secondary antibody, rat splenocytes were incubated with FITC-conjugated goat anti-mouse IgG2α mAb, the secondary stain for primary RT6 antibody. The upper panel shows an example of the scatter plot of cells in this preparation with the gate selected for lymphocytes indicated by the polygon shown between 200 and 400 on the forward scatter axis. The lower plot indicates the staining seen among cells treated with the secondary antibody, with the secondary antibody only seen in the FL1 channel since there is no FL2 signal present. Since no specific staining of these cells is possible, all cells in the FL1-FL2 plot are considered to be negative. RT6 positive cells will therefore be found to the right of the FL1 quadrant marker.

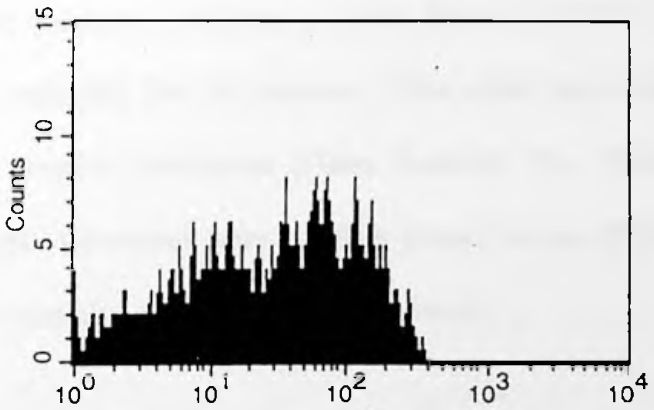




FL1: FITC labeled 2° mAb for RT6 staining of GT untreated control sample V2

Figure 8. Example of mean channel number calculation for $RT6^+$ cell surface markers. To evaluate the intensity of $RT6^+$ staining on spleen lymphocytes, rat splenocytes were first incubated with mouse anti-rat RT6 primary antibody, then visualized by FITC-conjugated mouse anti-rat RT6 mAb. The upper panel shows the scatter plot of cells in this preparation with the gate selected for lymphocytes as indicated by the polygon shown between 200 and 400 on the forward scatter axis. The lower histogram plot indicates the number of cells (y axis) with a given fluorescent intensity (x axis) seen among gated cells. The average fluorescent intensity of the analyzed cell population appear in the table below the histogram.





FL1: RT6 Staining

Total events	Gated events	Mean channel
10,000	1191	53.92

Deerfield, IL) at -20°C to a thickness of 10 microns. The sections were laid on Vectabond reagent (Vector Laboratory Inc., Burlingame, CA) coated slides. The slides were stored at -20°C overnight. After bringing slides to room temperature, they were fixed by immersion in acetone for 5 minutes at room temperature.

After washing with PBS, the slides were incubated in a humidified chamber at room temperature for 20 minutes covered with 1:50 dilution of horse serum (Vector Laboratory Inc., Burlingame, CA) to block nonspecific antibody binding. The tissue sections were then covered with fluorescent labeled specific primary antibody (1:1000 dilution of mouse anti-rat monoclonal antibody) at room temperature for 30 minutes in a humidified chamber. After staining, the slides were rinsed with distilled water. Since mouse anti-rat RT6 mAb is unlabeled, the tissue sections stained for RT6 were subsequently incubated with secondary antibody (1:1000 dilution of FITC labeled rat absorbed-goat anti-mouse antibody) for 30 minutes. The slides were placed on a horizontal surface and two drops of Gel/Mount (Fisher Scientific Co. Pittsburgh, PA) applied on the tissue sections. Coverslips were carefully placed on top of the mounting fluid, and the borders of the coverslip were sealed with permount.

Functional Evaluation

Leukocyte proliferation

As with the earlier experiments, lymphocytes for *in vitro* study were isolated from spleens of 65-day-old GT untreated DP/BB rats and treated with 1 µg/ml GT in tissue culture flasks for 1 hour prior to proliferation testing. Lymphocytes from the *in vivo* study were isolated from GT treated DP/BB rat spleen and their corresponding controls which

had been treated with vehicle only. Splenic lymphocytes were stimulated with ConA and proliferation was measured by a dye-reduction assay. Briefly, isolated splenic leukocyte suspensions were centrifuged in 15 ml test tubes in 500 x g for 4 minutes at 4 °C. Cell pellets were resuspended in complete MEM-10 (Sigma, St. Louis, MO). Leukocyte suspension and ConA solution were added to a 96-well microtiter plate (10^5 cells/well) with three wells for each sample. Cells in control wells were incubated with MEM medium only. The final concentration of ConA was 10 μg/ml. Alimar Blue (50 μl/well) (Sensititre/Alamar, Sacramento, CA) was then added to each of these wells. Samples in microtiter plates were incubated in a humidified 37 °C, CO_2 (5%) incubator for 72 hours. Proliferation test data were obtained from a microplate reader set to measure absorbance at 590 nm. The average absorbance of triplicate wells was used to calculate a stimulation index (SI) where SI = (Absorbance of sample with mitogen stimulation)/(Absorbance of sample without mitogen stimulation). The data were statistically analyzed by t test.

Apoptosis

Splenic lymphocytes isolated from GT treated DP/BB rats, controls, or splenic lymphocytes treated with GT *in vitro* and control lymphocytes were stained by the TUNEL method (terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-labeling) with Oncor Fluorescent ApopTag Kit (Oncor, Inc, Gaithersburg, MD) and analyzed by Becton Dickenson FACScan flow cytometry to determine the proportion of lymphocytes undergoing apoptosis. The TUNEL procedure measures elevated levels of free 3'-hydroxy DNA fragments.

In the TUNEL method, lymphocyte DNA is first tailed by terminal deoxynucleotidyl transferase (TdT) to add digoxigenin-deoxyribonucleotide triphosphate to the 3'-OH ends of fragmented double or single stranded DNA. Then digoxigenin-11-dUTP and dATP are bound by anti-digoxigenin antibody conjugated with FITC. All lymphocytes were counterstained by propidium iodide (PI).

To conduct the apoptosis analysis, 4 x 10^6 isolated leukocytes were recovered by centrifugation (500 x g) and fixed in 5 ml ice-cold 1% Paraformaldehyde in PBS at pH 7.4 for 15 minutes. The fixed leukocytes were washed three times with PBS. The cells were recovered by centrifugation at 1000 rpm in 5 minutes. The fixed leukocytes were resuspended in 70% ice-cold ethanol and kept at -20°C up to 5 days.

For each sample, 2 x 10^6 cells per sample were recovered by centrifugation and resuspended in 1 ml PBS. After two washes with PBS, the cells were resuspended in two drops of proprietary equilibration buffer. The cells were spun down and resuspended in 50 µl of working strength TdT enzyme, and incubated in a water bath for 30 minutes at 37°C. the reaction was stopped by adding 1 ml stop/wash buffer directly to the cell suspension. Cells were then washed twice with the buffer, resuspended in 100 µl of working strength anti-digoxigenin-fluorescein and incubated for 30 min at room temperature. After incubation, the cells were washed twice by 1.0 ml of 0.1% Triton X-100 in PBS. The cells were stained by 1.0 ml of propidium iodide staining solution for 15 minutes at room temperature in the dark.

Green fluorescence of anti-digoxigenin-fluorescein was measured at 510-550 nm by the flow cytometer while red fluorescence of PI was measured at >620 nm.

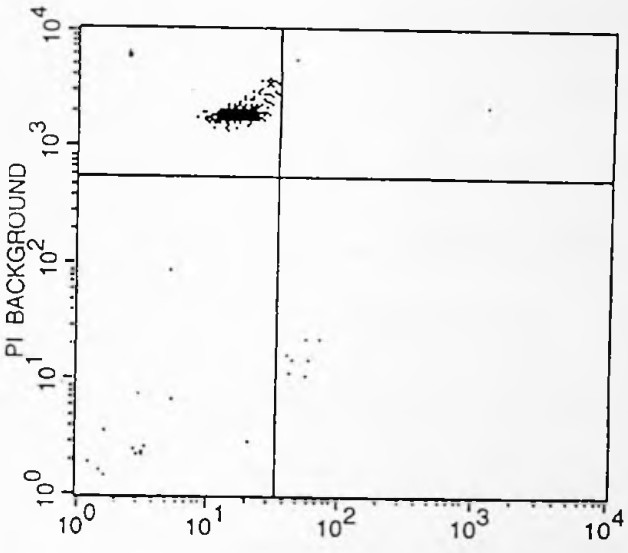
For analyses of apoptosis data, we first gated the lymphocyte populations from FSC versus SSC dot plots then replotted them as FITC (FL1) versus PI (FL2) plots. To establish the fluorescent intensity that identifies apoptotic vervus non-apoptotic cells, cell suspension were divided and stained by methods to produce positive and negative controls. The positive samples were stained with the complete stain (TUNEL procedure with FITC; counter stain with PI) and the negative controls were stained with counter stain (TUNNEL procedure used but with omission of FITC conjugated anti-digoxigenin antibody plus PI counter stain). These cells were analyzed by flow cytometry as shown in Figure 9 which indicates the level of FL1 staining associated with apoptotic cells. The intensity of the FL2 channels indicates PI staining associated with all cells. The highest intensity level of each parallel negative control sample was regarded as the lowest level consistent with a positive apoptosis staining reaction. The dots with greater staining intensity beyond this level was considered to represent apoptotic cells. The percentage of cells showing apoptosis was determined and comparisons made as noted in the results.

Statistical Analysis

Analysis of the incidence and onset of IDDM in DP/BB rats was performed using life-table analysis (Ingelfinger, 1987). All other comparisons between GT treated samples and controls as well as proliferative and apoptosis data yielded continuous data was therefor evaluated by student's t-test using a computerized statistical program in Sigma Plot (Jandel corporation, San Rafael, CA).

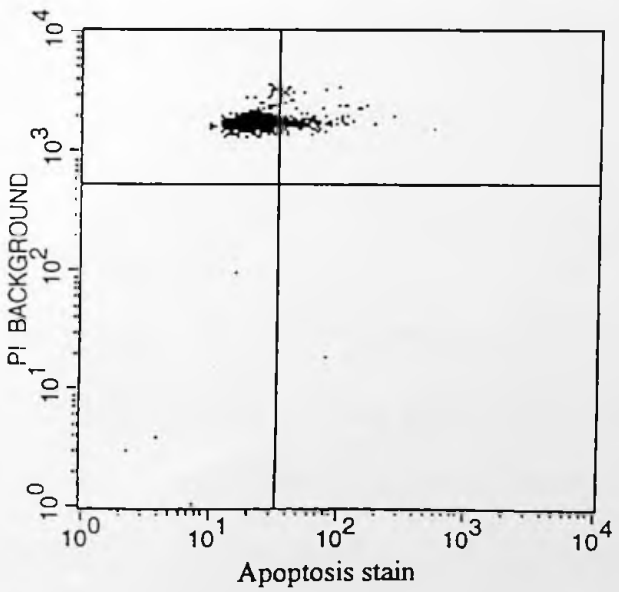
Samples for the *in vitro* portion of the test used aliquots of identical cell preparations allowing a paired t-test for the evaluations. The *in vivo* portion of the study

Figure 9. Sample data to illustrate counting of apoptotic cells. Lymphocyte samples containing apoptotic cells were treated with the complete stain (TUNEL procedure with FITC; counter stained with PI) and the negative controls were prepared by exposure to the counter stain only. The % of cells showing apoptosis (FITC staining) was determined by flow cytometry with 10,000 cells analyzed. The highest FL1 intensity level of each parallel negative control sample (shown in the upper panel) was regarded as the highest level consistent with negative apoptosis staining (shown in lower panel). The quadrant was drawn at the highest intensity level of each negative control sample. The cells located in upper-right quadrant of the lower panel were counted as apoptotic lymphocytes.



Total events: 10,000
Gated events: 1332
Quad location: 32,538

Quad	Events	% Gated
UL	1311	98.42
UR	1	0.08
LL	13	0.98
LR	7	0.53



Total events: 10,000
Gated events: 2494
Quad location: 32,519

Quad	Events	% Gated
UL	2287	91.70
UR	201	8.06
LL	5	0.20
LR	1	0.04

was derived from rats which had no one to one corespondance between GT treated and control animals. Therefore an unpaired t-test was used for *in vivo* derived data.

ANOVA was used to evaluate insulin release data and was performed using PC-SAS.

RESULTS

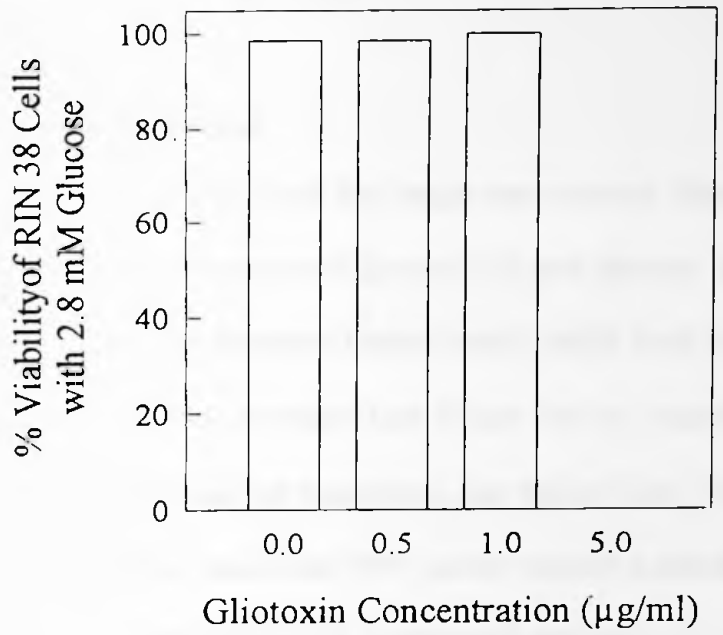
Toxicity of Gliotoxin

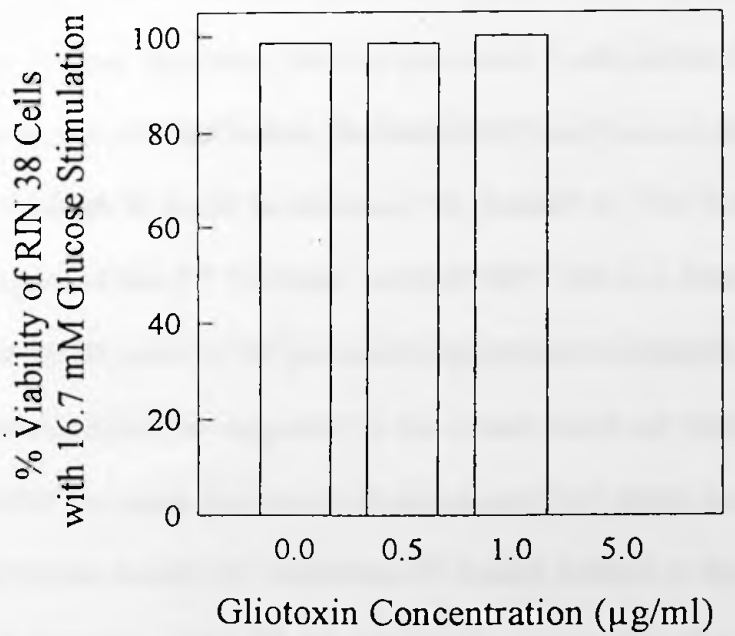
Early studies in our laboratory and in other laboratories indicated that GT is an immunomodulator with immunosuppressive functions. Although we wished to study GT as an immunotherapeutic/immunopreventive compound, it was essential to determine if doses which may exhibit beneficial effects on an autoimmune processes and prevent autoimmune diseases also produced unacceptable toxicity. Previous studies showed that gliotoxin at >100 μg /mouse produced lethal effects (Eichner, et al., 1986). However we have shown that concentrations as low as 1 μg/ml affected phagocytic activity of PMN. To predict what level of GT exposure might suppress lymphatic cells without destruction or enhancement of damage to insulin-secreting cells, GT toxicity was evaluated by several methods including its effect on cultured cells as well as its effect on intact animals.

RIN 38 cell viability

As shown in Figure 10, GT concentrations of 0.5 and 1 μg/ml did not alter the viability of RIN 38 cells. GT at 5 μg/ml was lethal for RIN 38 cells. The concentration of glucose in the incubation medium had no appreciable effect on RIN 38 viability as shown by cells exposed to GT in the presence of 2.8 mM (Figure 10, upper panel) or 16.7 mM (Figure 10, lower panel) glucose. The higher glucose concentration was neither protective for high GT doses nor did it enhance toxicity of lower GT concentrations. Because 1

Figure 10. Viability of RIN 38 cells after exposure to GT. RIN 38 insulinoma cells were cultured for 48 hours in RPMI-1640 with 5% FBS and 2.8 mM glucose at 37°C in a CO_2 (5%) incubator. After preincubation in Krebs-Ringer buffer (KRB) for 1 hour, the cells were incubated with GT (0, 0.5, 1 and 5 μg/ml) with low glucose concentration (2.8 mM, upper panel) or high glucose concentration (16.7 mM, lower panel) for 1 hour. All conditions were tested in triplicate. The viability of RIN 38 cell counts were determined by trypan blue staining. The columns indicate the percentage of viable RIN 38 cells. Standard errors did not exceed 0.2% of the mean values.


% Viability of RIN 38 Cells with 2.8 mM Glucose
100
80
60
40
20
0
0.0
0.5
1.0
5.0
Gliotoxin Concentration (µg/ml)


% Viability of RIN 38 Cells with 16.7 mM Glucose Stimulation
100
80
60
40
20
0
0.0
0.5
1.0
5.0
Gliotoxin Concentration (µg/ml)

μg/ml GT had previously been shown to affect PMN function, we were interested to know if an equivalent dose would be tolerated by β cells.

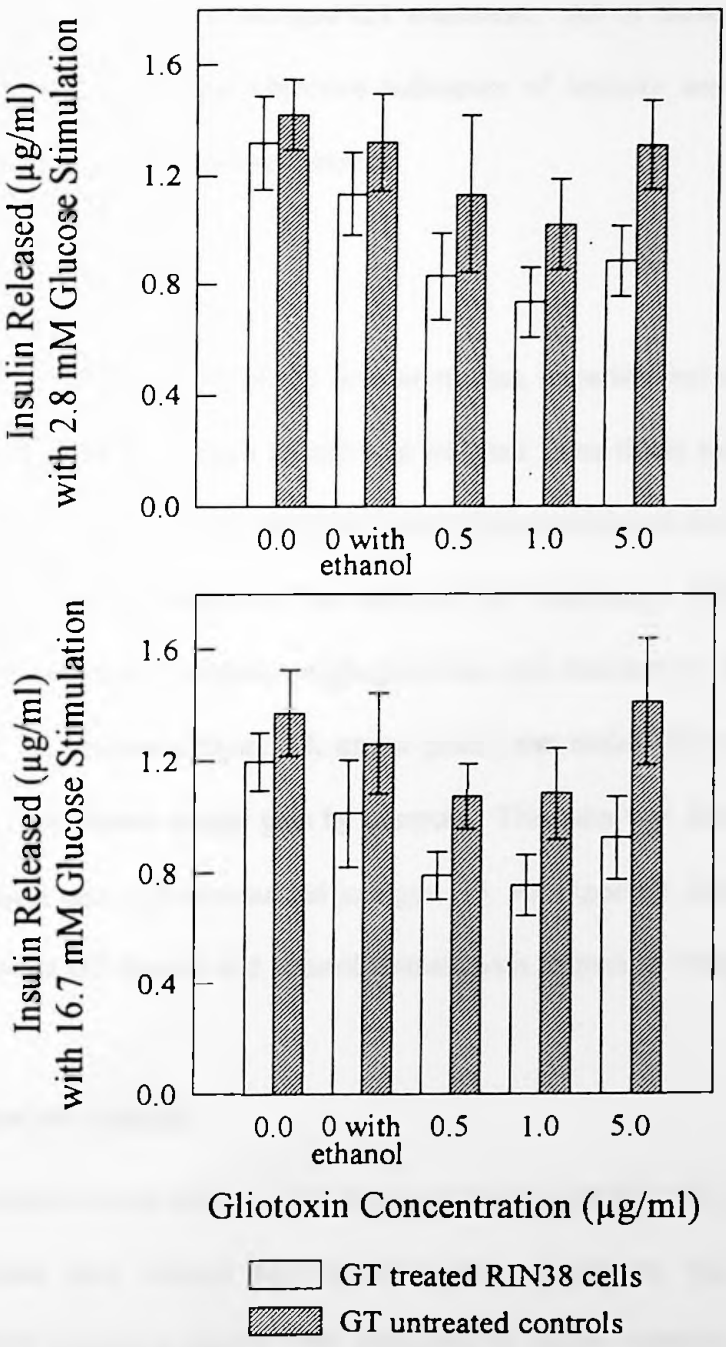
RIN 38 cell insulin secretion

GT treated RIN 38 cells produced less insulin than controls (Figure 11). RIN 38 cells were incubated in 2 concentrations of glucose (2.8 mM glucose, upper panel; 16.7 mM glucose, lower panel) and provided similar results under both conditions. Cells treated with 5 μg/ml GT were nonviable (see Figure 10) so insulin release at this concentration from these cells was not physiologic, but due to lysis. Although 1 μg/ml GT decreased insulin secretion, more than 70% insulin remains available and would not likely eliminate insulin production if given to experimental animals.

From these results, we reasoned that a dose equivalent to 1 μg/g of body weight would probably not cause significant harm to pancreatic ß cells either in terms of ß cell viability or function, but as noted before, this level of GT may have an effect on lymphoid cell function. Although it would be necessary to conduct *in vivo* evaluation, the cell culture results suggested that GT treatment would probably not be a cause of diabetes.

Treatment of BB rats with GT gave us the opportunity to attempt to document any evidence of systemic toxicity as suggested by the overall health of treated animals. The animals in this initial trial were given up to 36 injections of GT which provided an opportunity to note any gross toxicity by comparing GT treated animals to those given control (glycerin vehicle) injections. We did not observe any untoward effects of chronic GT treatment. Among those animals not developing diabetes, both control and GT-treated animals appeared equally healthy. Fur remained sleek and unruffled, the animals were not

Figure 11. Effect of GT on insulin secretion by RIN 38 cells. Insulin release from RIN 38 cells was measured by RIA with low glucose medium (upper panel) and high glucose medium (lower panel). Mean insulin values for culture with 0-5 μg/ml GT are shown; error bars represent standard error of the mean. Each condition was tested in sextuplicate culture after 1 hour of GT incubation. ANOVA for low glucose conditions (general linear models procedure for completely randomized 2-factor ANOVA) showed marginal non-significance ($p=0.069$). Because these data approached significance, a Tukey HSD (honestly significant difference) test was performed as a follow up. Tukey HSD showed that at $p=0.05$ the controls with or without ethanol were not significantly different but insulin secretion at each of the 3 GT test concentrations differed from their respective controls. Similar results were obtained for high glucose concentration.


Insulin Released (µg/ml)
with 2.8 mM Glucose Stimulation
1.6
1.2
0.8
0.4
0.0
0.0
0 with ethanol
0.5
1.0
5.0
Insulin Released (µg/ml)
with 16.7 mM Glucose Stimulation
1.6
1.2
0.8
0.4
0.0
0.0
0 with ethanol
0.5
1.0
5.0
Gliotoxin Concentration (µg/ml)
GT treated RIN38 cells
GT untreated controls

lethargic, eyes and mucosa appeared normal, food and water consumption were unaffected, and there was no other evidence of cumulative toxicity (diarrhea, neurologic signs for example) associated with prolonged GT treatment. All of these observations, however, were subjective. Several objective indicators of toxicity were recorded for GT treated rats and their corresponding controls.

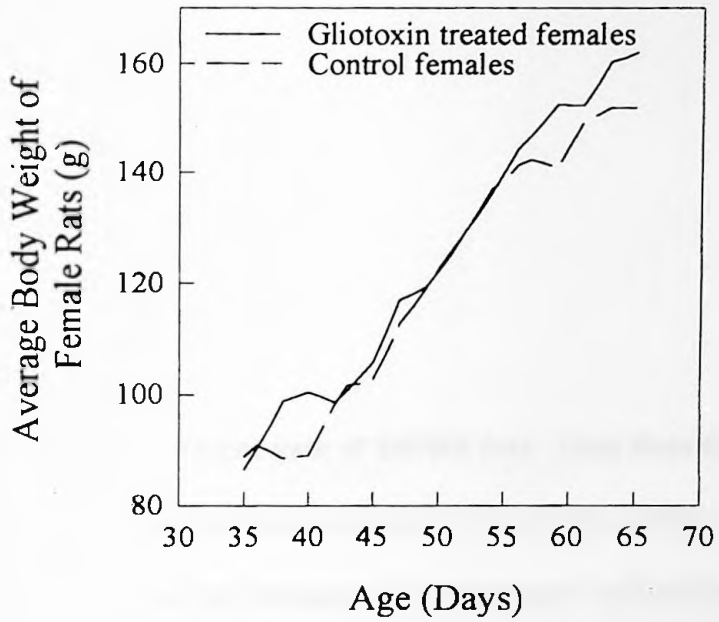
Effect on body weight

During the initial month of the *in vivo* studies, experimental animals were young and rapidly gaining weight. Each animal was weighed three times weekly corresponding to the times when GT or control injections were administered and the rate of weight gain was used as an objective measure of the safety of GT treatment. Because males and females attain different body weights, weight gain data was analyzed by gender. The history of weight gain for females (Figure 12, upper panel) and males (Figure 12, lower panel) treated with GT paralleled weight gain by controls. This data was further analyzed as average daily weight gain and summarized in Figure 13. As expected, there was no statistical difference between GT treated and control animals with respect to weight gain.

Leukocyte counts

One concern about the use of immunosuppressive drugs is the possibility that they may compromise host defense against infections. Therefore, the effect of GT on peripheral blood leukocyte counts was measured in blood samples from non-diabetic DP/BB rats treated with GT (1 μg/g of body weight) three times weekly for a total of 10 doses and compared to blood counts from control animals injected with vehicle on an

Figure 12. Average body weight of DP/BB rats treated with GT or control injections. Five female and 5 male DP/BB rats were treated with GT (1μg/g body weight, intraperitoneal injection) three times weekly beginning at 30 days of age. The same number of rats received vehicle (glycerin) only. The weight was recorded for each animal from 30 to 65 days of age and the average weight of each group was plotted.


Gliotoxin treated females
Control females
Average Body Weight of Female Rats (g)
160
140
120
100
80
30 35 40 45 50 55 60 65 70
Age (Days)

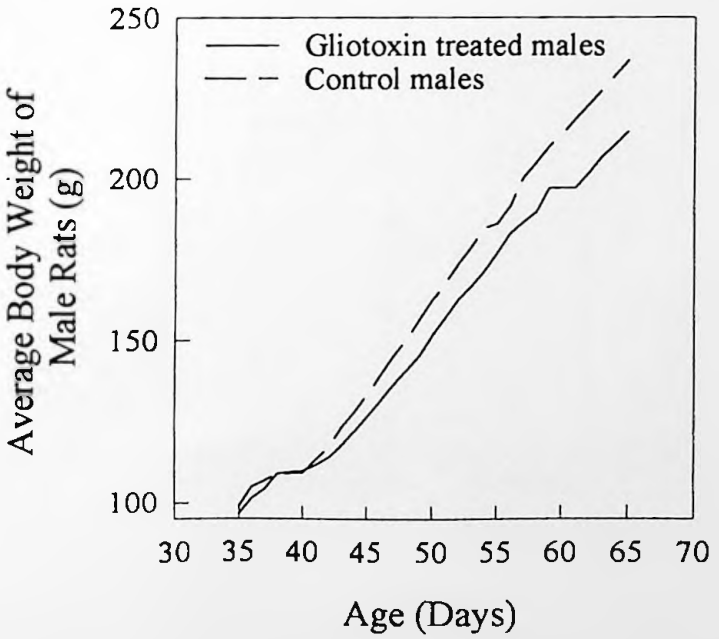
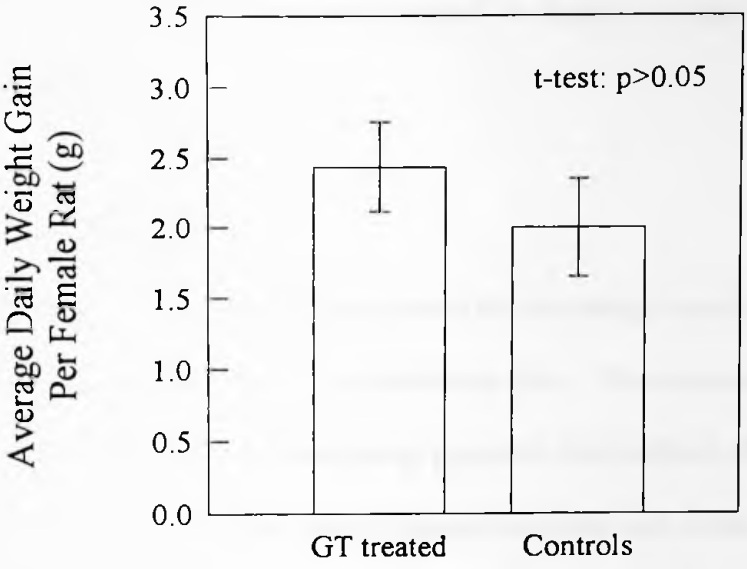
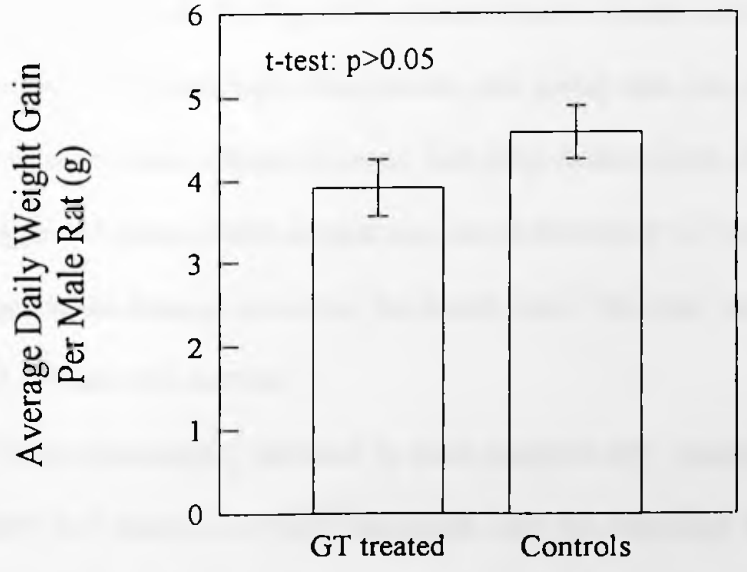

Gliotoxin treated males
Control males
Average Body Weight of Male Rats (g)
250
200
150
100
30 35 40 45 50 55 60 65 70
Age (Days)

Figure 13. Average daily weight gain of DP/BB rats. Data from figure 11 was used to calculate the average weight gain for each group (5 rats per group) expressed on a daily basis. The columns indicate the average daily weight gain with the standard error of the mean shown. Statistical comparison employed unpaired t-test.


Average Daily Weight Gain
Per Female Rat (g)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
t-test: p>0.05
GT treated
Controls


Average Daily Weight Gain
Per Male Rat (g)
6
5
4
3
2
1
0
t-test: p>0.05
GT treated
Controls

identical treatment schedule. These data were reported by gender (Figure 14). The leukocyte counts of GT treated and control in female or male DP/BB rats were not statistically different.

Histopathology

The liver provides an efficient system for extracting toxins from blood resulting in their catabolism, storage, and/or excretion into bile. The sequestration of toxins by the liver makes it a key organ for evaluating potential toxic effects of GT. The portal tract contains a branch of the portal vein, a hepatic arteriole, and a bile duct. Blood entering the portal tract through the portal vein and hepatic artery is mixed in the penetrating vessels, enters the sinusoids and percolates along the cords of parenchymal cells (hepatocytes), eventually flowing into terminal hepatic venules, and exits the liver through the hepatic vein. In histologic observations, the portal area may be the most important region to observe toxic effects of toxins, including those related to GT treatment (Mary, 1993). Figure 15 shows H&E stained sections of liver from GT treated and control non-diabetic rats taken from an area near the portal tract. No clear morphological difference was noted in these two sections.

Tissue regeneration, indicated by multi-nucleate cells, signifies cellular response to toxic effects and examples of liver binucleate cells are identified in Figure 15. Cellular vacuolization also indicates toxicity. In the observation of GT toxicity, binucleate cells and cell vacuolization in the portal area of the liver were recorded in GT untreated, non-diabetic DP/BB rat liver sections and GT treated non-diabetic DP/BB rat liver sections. Figure 16 shows the number of binucleate cells counted in the portal area in liver sections.

Figure 14. Peripheral leukocyte counts in GT treated and control rats. BB rats (4 males and 4 females) were treated with GT (1μg/g body weight, intraperitoneal injection) from 30 days of age and the same number of rats received vehicle (glycerin) only as controls. After 10 GT injections, 20 μl of blood was collected from the tail with Unopette microcollection system. After lysis of red cells, the leukocytes were counted microscopically. The columns indicate the leukocyte counts and error bars show the standard error of the mean. Male and female data is shown separately because male rats typically have higher absolute white blood counts than females.

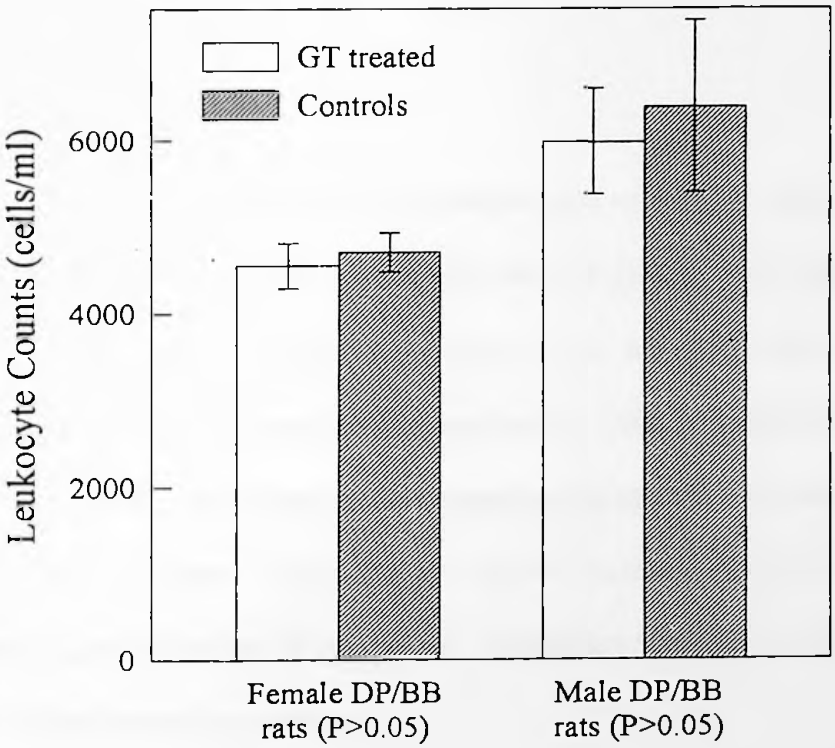
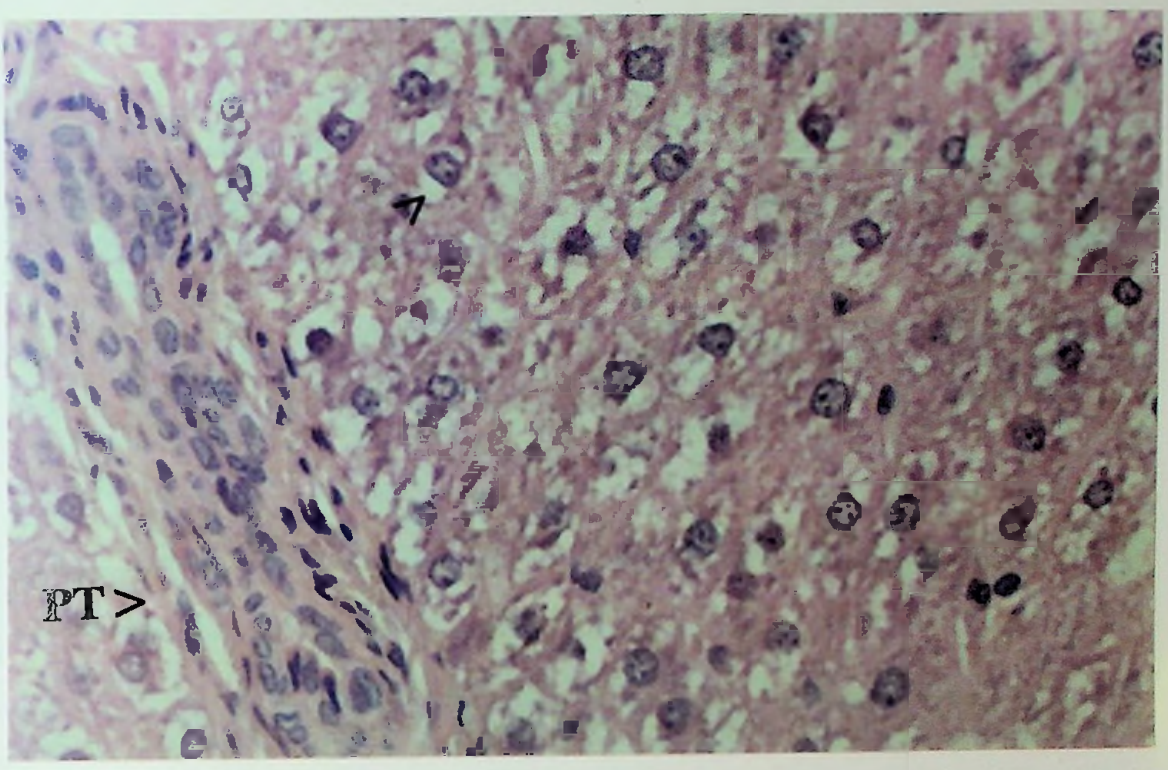

GT treated
Controls
Leukocyte Counts (cells/ml)
6000
4000
2000
0
Female DP/BB rats (P>0.05)
Male DP/BB rats (P>0.05)

Figure 15. Liver morphology of GT treated and control nondiabetic DP/BB rats. DP/BB rats were injected three times weekly with GT (1μg/g body weight, intraperitoneal injection) from 30 days of age to 90 days of age and euthanized prior to diabetes development. The livers were removed and fixed in 10% formalaldehyde then embedded in paraffin. The 10 micron liver sections were cut and stained with hematoxylin and eosin (H&E). The upper panel is the portal area of liver section (100x) from a control rat and the lower panel is from a GT treated rat. Exemplary binucleate cells are marked with arrows. PT indicates the portal tract.


PT>

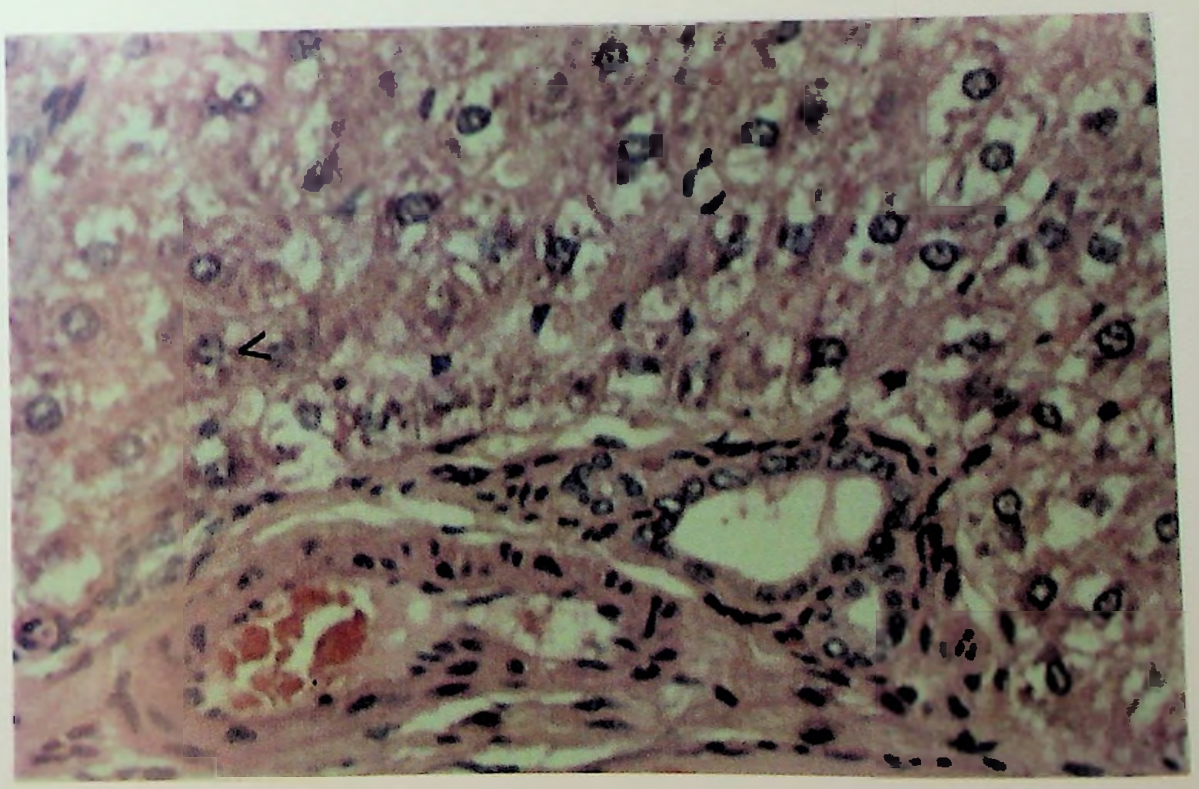
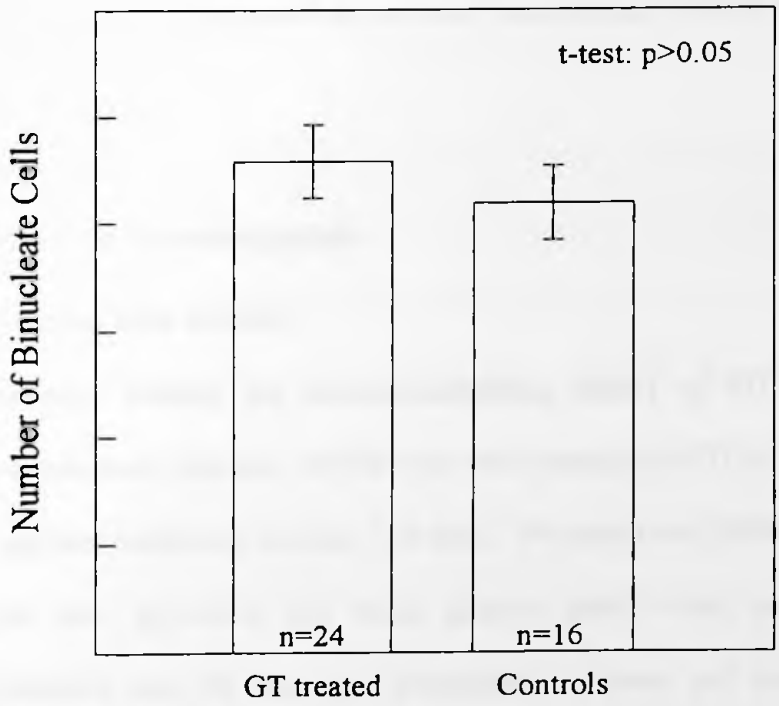


Figure 16. Prevalence of binucleate cells in liver sections from GT treated and control DP/BB rats. H&E stained liver sections (c.f. Figure 15) were evaluated for binucleate cells after GT or control injections. The binucleate cells in portal regions of liver sections which reflect the degree of regeneration in response to hepatic damage, were counted in 8 high-power fields per rat. The columns indicate the number of fields counted "n"; the average number of binucleate cells in each microscopic field (400x); and error bars show the standard error of the mean.


t-test: p>0.05
Number of Binucleate Cells
n=24
n=16
GT treated
Controls

No difference in this measure of toxicity was found. No clear evidence of vacuolization was seen in GT treated animals.

Since various toxins and drugs may be concentrated and excreted by kidney, it also provides an important organ for evaluation of systemic toxicity. Typical signs of glomerulus toxicity include protein precipitation, cell regeneration and cell vacuolization. We did not see any increase of these characteristics in GT treated non-diabetic DP/BB rat kidney sections (Figure 17) compared to untreated non-diabetic DP/BB rat kidney sections.

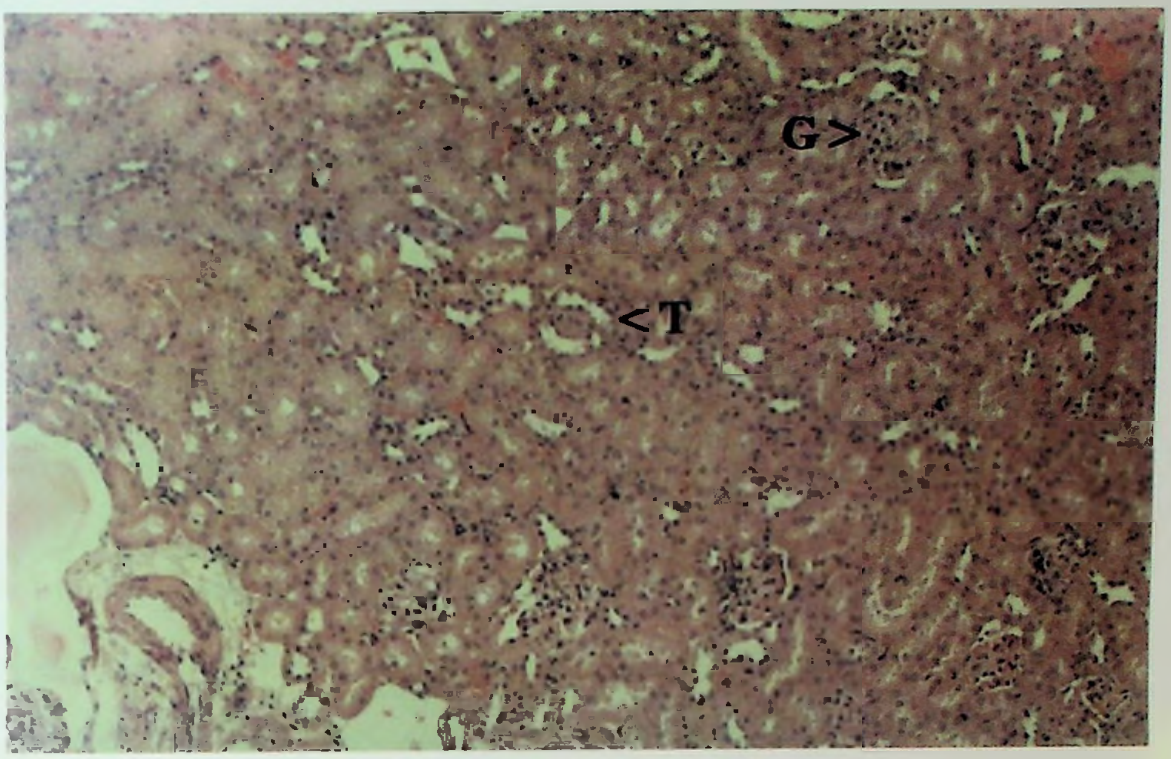
Gliotoxin Effect on Diabetogenesis

Incidence and time of onset

To determine whether the immunomodulating effects of GT can influence progression of autoimmune diabetes, DP/BB rats were treated with GT or vehicle starting at 30 days of age and continuing through 120 days. We monitored IDDM onset on the basis of weight loss, glycosuria and blood glucose levels when necessary. Our observations indicated that GT treatment diminished incidence and delayed onset of IDDM pathogenesis if begun at 30 days of age.

As shown in Figure 18, 9 of 10 GT untreated control rats turned diabetic between age 60-120 days. This 90% diabetes incidence was comparable to the 86% average incidence of diabetes among DP/BB rats reported in the literature. Only 5 of 9 GT treated experimental DP/BB rats developed diabetes. When these data were evaluated by means of life table analysis, GT treatment proved significantly protective. GT treatment started from the age of 40 days was not protective Figure 19.

Figure 17. Kidney morphology of GT treated and control nondiabetic DP/BB rats. DP/BB rats were injected three times weekly with GT (1μg/g body weight, intraperitoneal injection) from 30 days of age to 90 days of age and euthanized prior to diabetes development. The kidneys were removed and fixed in 10% formalaldehyde then embedded in paraffin. The 10 micron kidney sections were cut and stained with hematoxylin and eosin (H&E). The upper panel is the section from a control rat and the lower panel is from a GT treated rat. "G" represents the glomerular structure and "T" represents renal tubules.


G>
<T

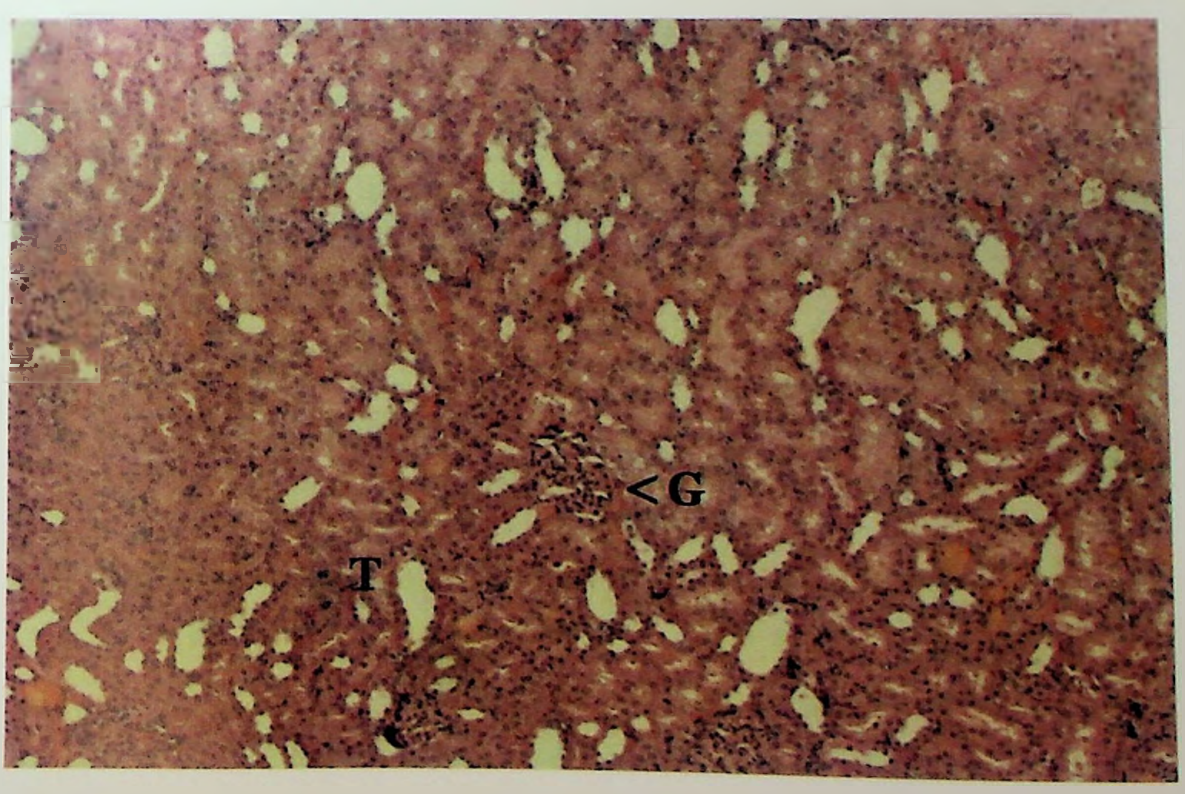

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Figure 18. Survival curves of GT treated DP/BB rats and untreated controls (Treatment started at 30 days of age). Two groups of DP/BB rats, 10 rats per group were injected with GT (1μg/g body weight, intraperitoneal injection) or with vehicle three times weekly beginning at 30 days of age through 120 days of age. The onset of diabetes was determined by weight loss, glycosuria, and hyperglycemia. Survival curves were compared using life table analysis.

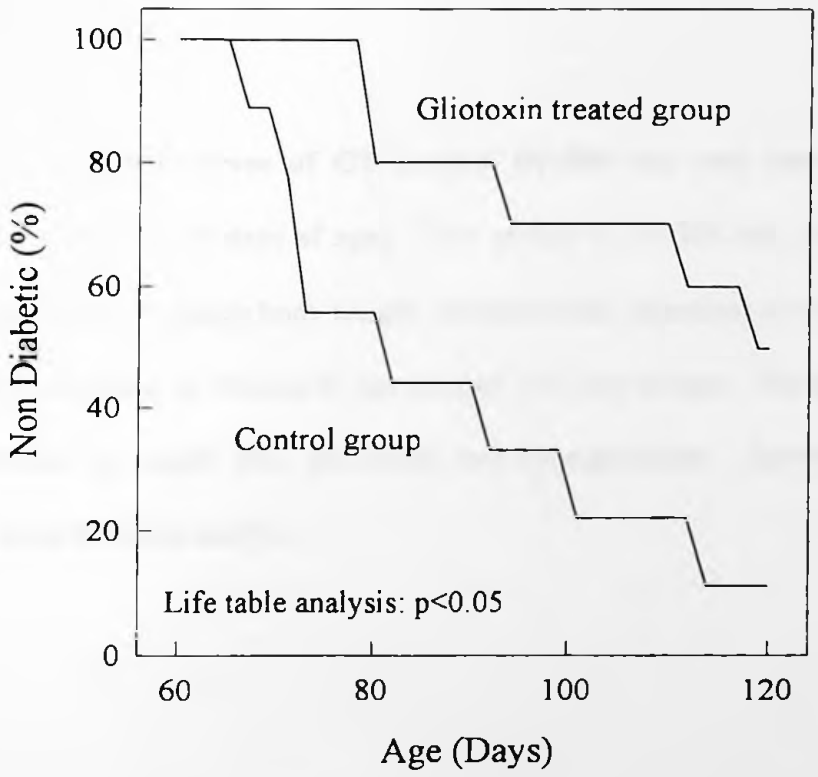
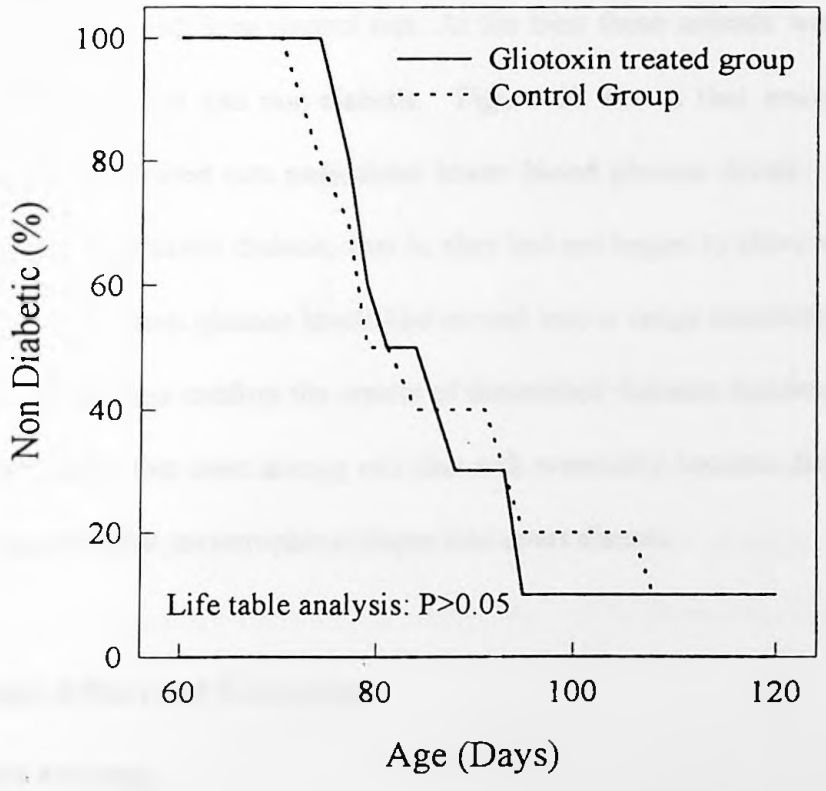

100
80
60
40
20
0
Non Diabetic (%)
Gliotoxin treated group
Control group
Life table analysis: p<0.05
60
80
100
120
Age (Days)

Figure 19. Survival curves of GT treated DP/BB rats and untreated controls (Treatment started at 40 days of age). Two groups of DP/BB rats, 10 rats per group were injected with GT (1μg/g body weight, intraperitoneal injection) or with vehicle three times weekly beginning at 40 days of age through 120 days of age. The onset of diabetes was determined by weight loss, glycosuria, and hyperglycemia. Survival curves were compared using life table analysis.


Gliotoxin treated group
Control Group
Non Diabetic (%)
100
80
60
40
20
0
Life table analysis: P>0.05
60
80
100
120
Age (Days)

Serum glucose

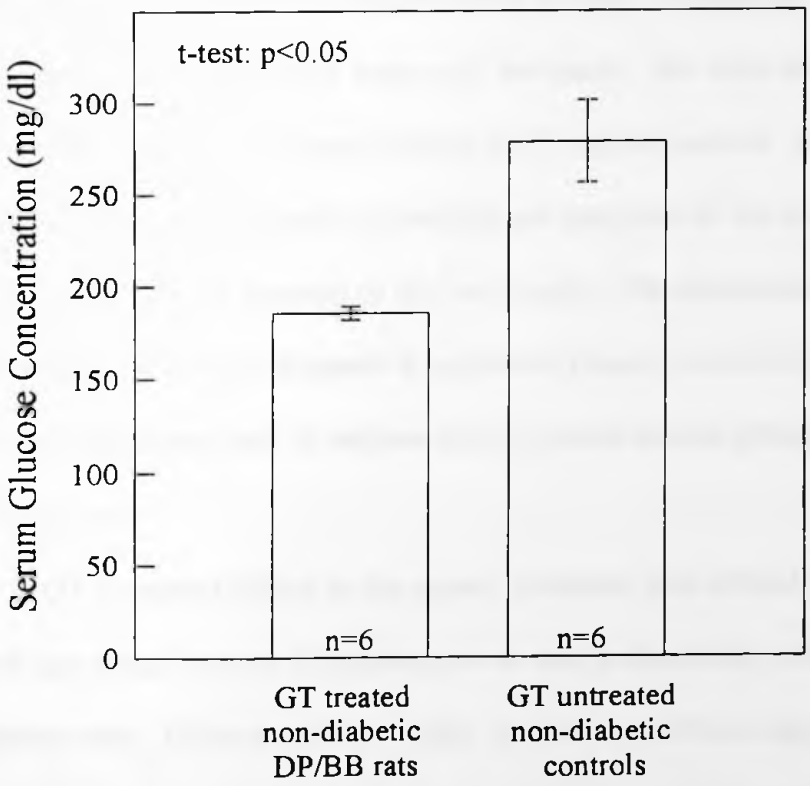
Elevated blood glucose is a hallmark of IDDM. Destruction of pancreatic islet β cells results in lower insulin production and reduced glucose metabolism. The primary diagnostic test for IDDM is measurement of the blood glucose concentration. Blood samples were taken from DP/BB rats that had been treated with GT three times a week beginning at 30 days of age and from control rats. At the time these animals were euthanized they were 90 days of age and non-diabetic. Figure 20 shows that among these pre-diabetic animals, GT treated rats maintained lower blood glucose levels. The GT untreated rats were not clinically diabetic, that is, they had not begun to show weight loss or glycosuria, but their serum glucose levels had moved into a range consistent with developing disease. These data confirm the results of diminished diabetes incidence among GT treated rats and imply that even among rats that will eventually become diabetic, the GT treatment postpones their catastrophic collapse into overt disease.

Immunologic Effects of Gliotoxin

Spleen histology

The spleen contains peripheral (secondary) lymphoid tissue. Its functions include filtration of blood and trapping blood-borne antigens and participation in the initial immune response to all invasive and particulate pathogens. The spleen is surrounded by a thin, glistening connective tissue capsule. The splenic white pulp consists of follicles which contain many T lymphocytes. The red pulp is organized with sinuses which are lined with macrophages, lymphocytes and plasma cells.

Figure 20. Effect of GT on serum glucose levels of prediabetic DP/BB rats. DP/BB rats were treated with GT (1μg/g body weight, intraperitoneal injection) three times weekly from 30 days of age to 90 days of age. The control group received injections of vehicle (glycerin) on a schedule identical to that used for GT-treated rats. Serum samples were collected from animals at 90 days of age and glucose concentrations measured with the glucose oxidase procedure. The bars indicate the mean serum glucose concentration, error bars show the standard error of the mean, and "n" indicates the number of rats tested.


t-test: p<0.05
Serum Glucose Concentration (mg/dl)
300
250
200
150
100
50
0
n=6
n=6
GT treated non-diabetic DP/BB rats
GT untreated non-diabetic controls

Histology of H&E stained spleen sections from GT treated pre-diabetic or non-GT treated prediabetic BB rats showed that GT treatment causes histologic changes which are primarily manifest in the T cell dominant white pulp. The splenic follicles appeared to have decreased prominence in GT treated rat spleen sections, compared to untreated controls as shown by Figure 21. The outer layer of the splenic follicle, ordinarily rich with T-cells, clearly decreased in GT treated spleen sections. Meanwhile, the red pulp area becomes more prominent as the white pulp decreases. We were able to morphometrically document the changes in splenic follicles in GT treated animals. Follicles were examined microscopically and the radius of medulla and thickness of the cortex measured directly from a video monitor attached to the microscope. The mathematical relationship of medullary radius to cortical thickness is reported in Figure 22 for 24 GT treated follicles from 3 GT-treated animals and 16 sections from 2 control animals among GT treated compared to control rats

GT produced effects in the spleen consistent with altered lymphocyte abundance which may either involve all lymphocytes or may preferentially influence only a subset of lymphoid cells. To have optimum utility, an immunopharmacologic agent should produce a selective effect. Therefore the next phase of this study investigated whether GT may have global or selective effects on lymphocyte subsets with particular relevance to IDDM.

Cells with MHC class II surface proteins

Autoantigen processing and presentation are important first steps in autoimmune disease pathogenesis, especially in organ specific autoimmune diseases such as IDDM. The loss of MHC II positive cells could provide an indicator of the loss of antigen pre-

Figure 21. Splenic follicle morphology of GT treated and control DP/BB rats. DP/BB rats were injected three times weekly with GT (1 μg/g body weight, intraperitoneal injection) from 30 days of age to 90 days of age and euthanized prior to diabetes development. The spleen samples were removed and fixed in 10% formaldehyde, then embedded in paraffin. The 10 micron spleen sections were cut and stained with hematoxylin and eosin (H&E). The upper panel shows a splenic follicle from a control rat and the lower panel shows a typical splenic follicle from a GT treated rat. The medulla in each photograph is indicated by vertical markers and the entire follicle (cortex and medulla) is marked by horizontal lines.

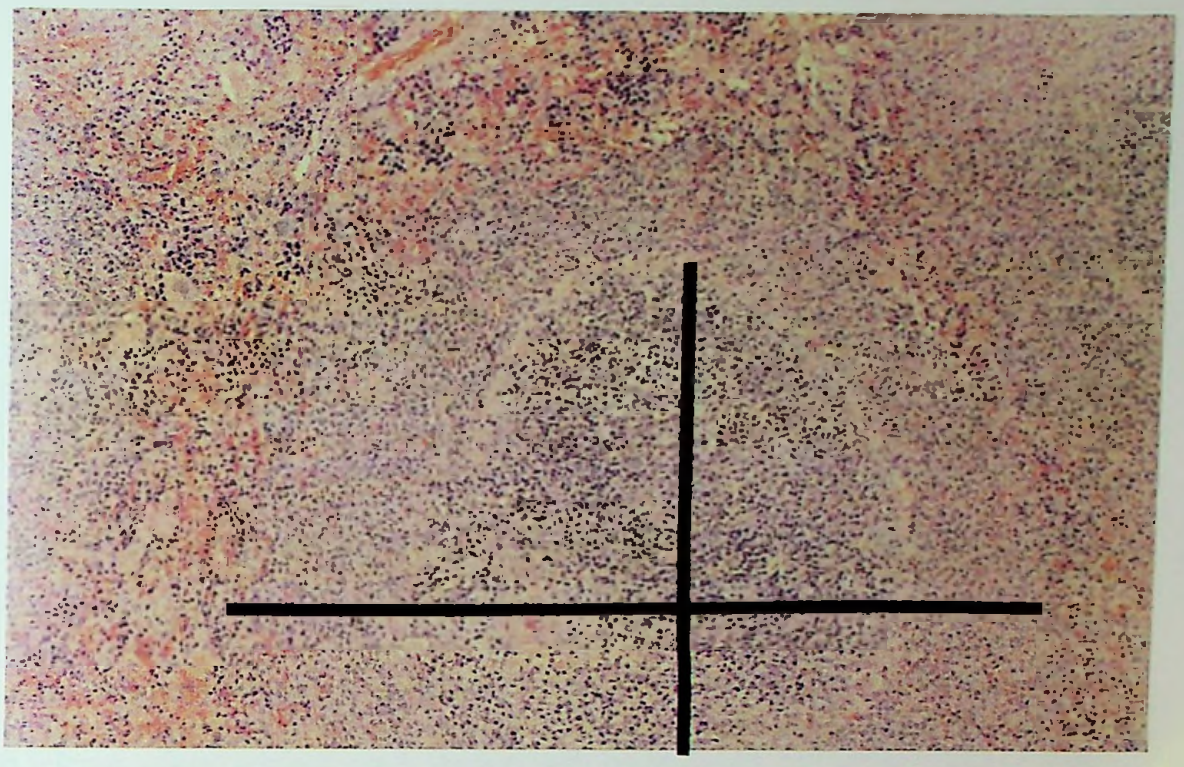
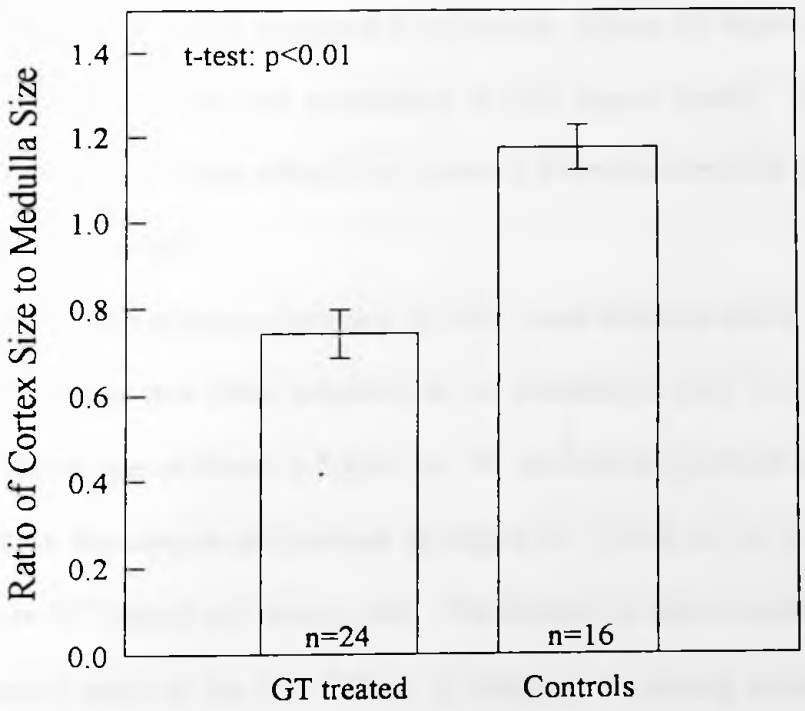


Figure 22. Effect of GT on splenic follicle morphology from DP/BB rats. DP/BB rats were injected three times weekly with GT (1 μg/g body weight, intraperitoneal injection) from 30 days of age to 90 days of age and euthanized prior to diabetes development. The spleen samples were removed and fixed in 10% formaldehyde, then embedded in paraffin. The 10 micron spleen sections were stained with hematoxylin and eosin (H&E). The cortical thickness and medullary radius of the splenic white pulp follicles were measured directly from a video image. Morphometric analysis was made from observation of 8 low-power fields from each rat. Data are represented as the thickness of the cortex divided by the radius of the medulla for each follicle. The columns indicate the mean value of the cortex-medulla ratio, error bars show the standard error of the mean, and "n" is the number of follicles counted.


Ratio of Cortex Size to Medulla Size
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
t-test: p<0.01
n=24
n=16
GT treated
Controls

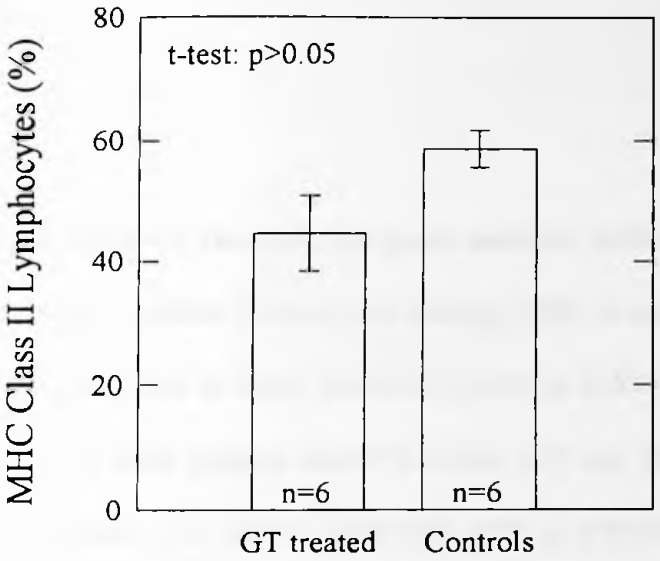
senting ability that may contribute to decreased autoimmune disease. The effect of GT on leukocytes with MHC II expression was investigated following *in vitro* GT treatment of splenocyte suspensions. Spleen cell preparations were incubated with FITC conjugated mouse anti rat MHC class II mAb and measured by flow cytometry. These cell suspensions were not pre-incubated in plastic flasks to remove adherent cells as subsequent studies of lymphocytes were. Initially flow cytometry data for MHC II stained cells was evaluated by gating on a region containing lymphocytes. Figure 23 shows that there was no significant effect of GT on this populations of cells (upper panel). Likewise ConA treatment (lower panel) of these cells did not reveal a distinction between GT treated cell suspensions and control cells.

Because a more relevant population of cells would be those which are larger and perhaps more granular than those contained in the lymphocyte gate, we reanalyzed the data with a different gate as shown in Figure 24. GT did have a significant effect on MHC II positive cells in this analysis as illustrated by Figure 25. ConA served to widen the difference between GT treated and control cells. The number of cells included in this larger gate was relatively small so that it is difficult to consider this finding conclusive with regard to antigen presentation.

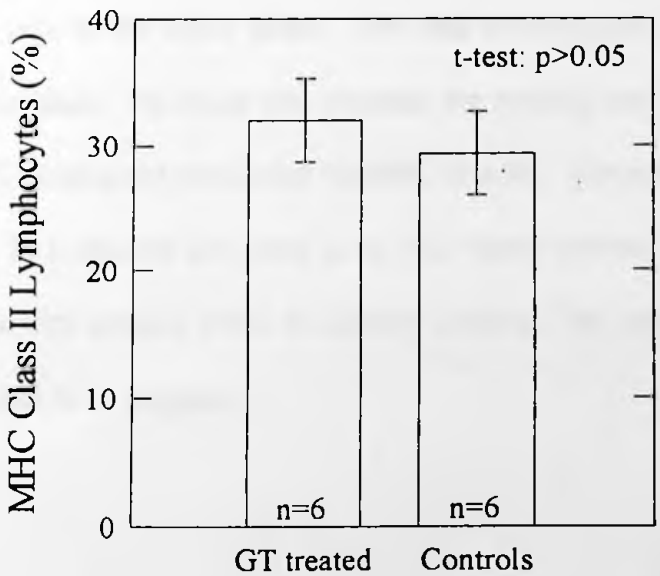
NK cells

Many studies indicate that NK cells are major attackers of pancreatic islet β cells (reviewed in introduction). If these cells are susceptible to GT then this would imply that some or all of the beneficial effects of GT may occur at the effector stage of IDDM pathogenesis.

Figure 23. Effect of GT on MHC II^+ DP/BB rat splenic lymphocytes exposed to GT *in vitro*. Spleen cell cultures were divided into 4 aliquots, and 2 aliquots were treated with GT (1 μg/ml) for 1 hour at 37°C in a CO_2 (5%) incubator and one GT treated aliquot was subsequently incubated with ConA (10 μg/ml) for 1 hour at 37°C in a CO_2 (5%) incubator. Half of the non-GT treated control cells were also stimulated with ConA and the remainder served as unstimulated controls. Cultures were stained with FITC-conjugated anti-MHC II antibody and staining evaluated by flow cytometry with 10,000 cells analyzed. Columns indicate the mean % of cells staining for the MHC II marker, error bars show the standard error of the mean, and "n" indicates the number of rats evaluated.

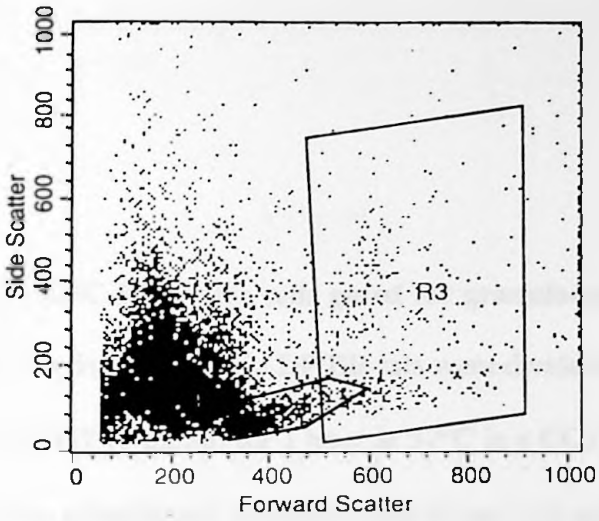


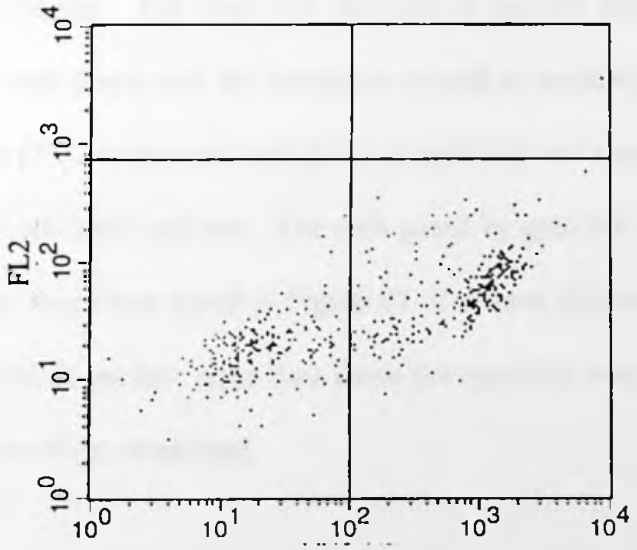
In vitro Study of
MHC Class II Splenic Lymphocytes



In vitro Study of MHC Class II Splenic
Lymphocytes with ConA Stimulation

Figure 24. Example to illustrate two different gates used for MHC II data analysis. To evaluate the percentage of spleen lymphocytes bearing MHC II surface markers, the lymphocyte population was gated as other lymphocyte subsets (CD8+, NK subsets for example). This gate is the small polygon shown between 300 and 500 on the forward scatter axis in the upper panel. To analyze large cells such as granulocytes, monocytes and macrophages, a wider gate was used (large polygon, upper panel) to include cells with higher granularity and larger size. This gate (R3) is shown between 500 and 900 on the forward scatter axis in the upper panel. The cells encompassed by gate R3 were replotted in the lower panel. The lower plot indicates the staining seen among gated cells treated with the FITC-conjugated mouse anti rat MHC II mAb. The antibody staining will only be seen in the FL1 channel and there is no FL2 signal present. The right lower quadrant shows cells with positive MHC II antibody staining. The cells in the left-lower quadrant are considered to be negative.

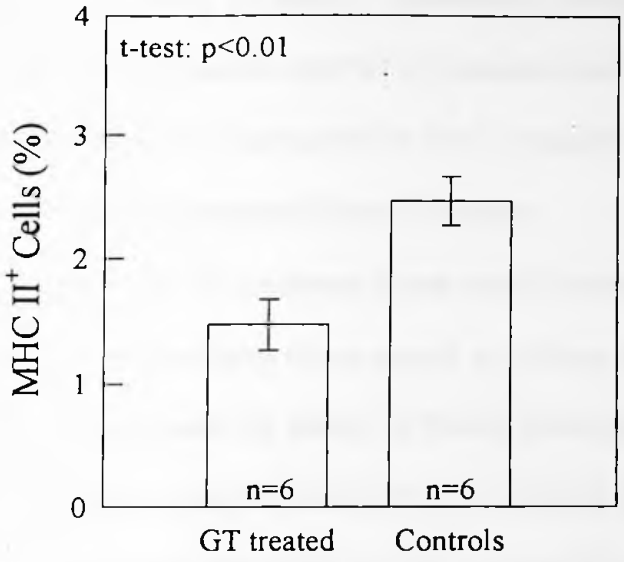




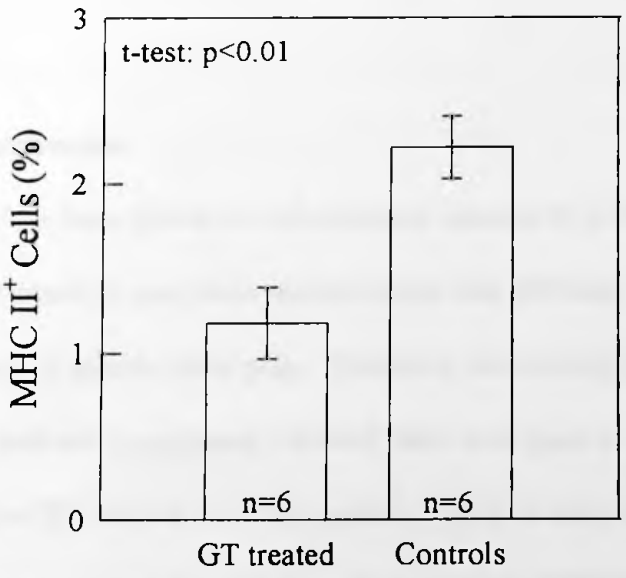
Total events:	10,000
Gate:	R3
Gated events:	471

Quad	Events	% Gated	% Total
UL	0	0.00	0.00
UR	1	0.21	0.01
LL	212	45.01	2.12
LR	258	54.78	2.58

Figure 25. Effect of GT on MHC II positive cells gated for granulocytes, monocytes and macrophages. Spleen cells from 65 day old DP/BB rats were divided into 4 aliquots. Two aliquots were treated with GT (1 μg/ml) for 1 hour at 37°C in a CO_2 (5%) incubator and one GT treated aliquot was subsequently incubated with ConA (10 μg/ml) for 1 hour at 37°C in a CO_2 (5%) incubator. The other two aluquots of the GT untreated control cells were also stimulated with ConA and the remainder served as unstimulated controls. Cultures were stained with FITC-conjugated anti-MHC II antibody and staining evaluated by flow cytometry with 10,000 cells analyzed. The cells gated by gate R3 in this analysis were the splenocytes larger than those gated in Figure 23. Columns indicate the mean % of cells staining for the MHC II marker, error bars show the standard error of the mean, and "n" indicates the number of rats evaluated.



In vitro Study of MHC II$^+$ Splenocytes



In vitro study of MHC II Splenocytes
with ConA Stimulation

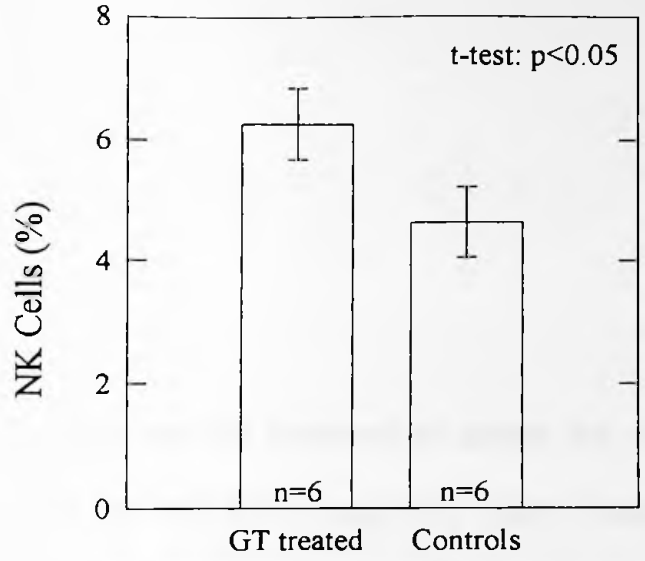
GT effects on NK lymphocytes were first studied *in vitro*. Splenic lymphocytes were isolated from GT untreated, 65 day-old, nondiabetic DP/BB rats. Cells were incubated for 1 hour with GT and labeled with FITC conjugated anti NK mAb. The percentage of FITC labeled lymphocytes was counted by flow cytometry. The experiments were performed with or without superimposed ConA stimulation.

As shown in Figure 26, GT treatment (upper panel) increased the relative NK cell percentage, but with ConA stimulation (lower panel), no difference was seen. These data suggested that GT does not exert its effects on IDDM through NK suppression. NK staining of lymphocytes from animals treated with GT compared to the untreated controls showed no difference regardless of whether the cell suspension was exposed to ConA or not (Figure 27). This further reduced the likelihood that NK cells represent the chief target of GT.

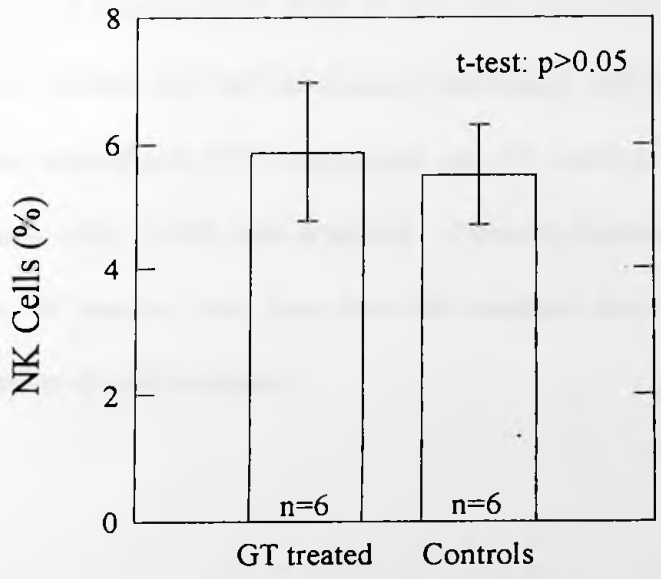
$CD5^+$ lymphocytes

Emphasis has been placed on cell-mediated damage in IDDM and less on humoral mechanisms. Furthermore our observations reflect that GT may have an effect on morphological changes of splenic white pulp. Therefore, we focused attention on T-cells and subsequently on their subpopulations. A PanT stain was used to evaluate the percentage of cells bearing the CD5 marker and was applied to both *in vitro* and *in vivo* experiments. Lymphocytes were recovered from 65 day old prediabetic DP/BB rats for *in vitro* study. These cells were treated with GT or vehicle and aliquots were stimulated with ConA or left unstimulated. A PE labeled mouse anti rat CD5 monoclonal antibody was used for flow cytometer enumeration of stained cells.

Figure 26. Effect of GT on DP/BB rat splenic NK cells exposed to GT *in vitro*. Spleen cells from 65 day old DP/BB rats were obtained and preincubated for 1 hour at 37°C in MEM plus 10% FCS in polystyrene flasks in a CO_2 (5%) incubator. Cultures were divided into 4 aliquots, and 2 aliquots were treated with GT (1 μg/ml) for 1 hour at 37°C in a CO_2 (5%) incubator and one GT treated aliquot was subsequently incubated with ConA (10 μg/ml) for 1 hour at 37°C in a CO_2 (5%) incubator. Half of the non-GT treated control cells were also stimulated with ConA and the remainder served as unstimulated controls. Cultures were stained with FITC-conjugated anti-NK antibody and staining evaluated by flow cytometry with 10,000 cells analyzed. Columns indicate the mean % of cells staining for the NK marker, error bars show the standard error of the mean, and "n" indicates the number of rats evaluated.

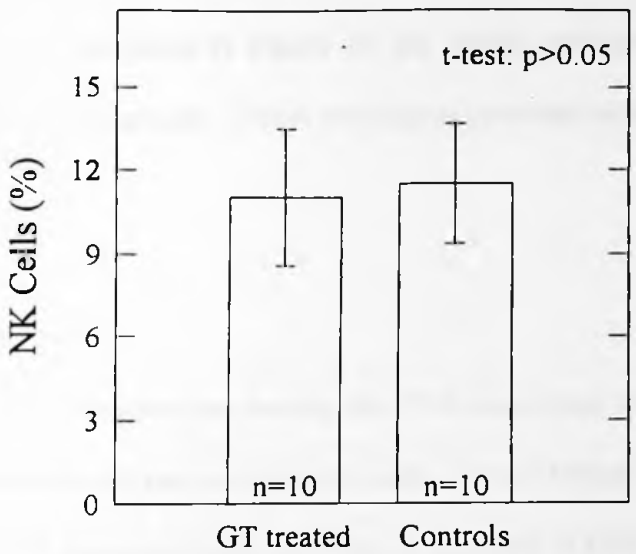


In vitro Study of Splenic NK Cells

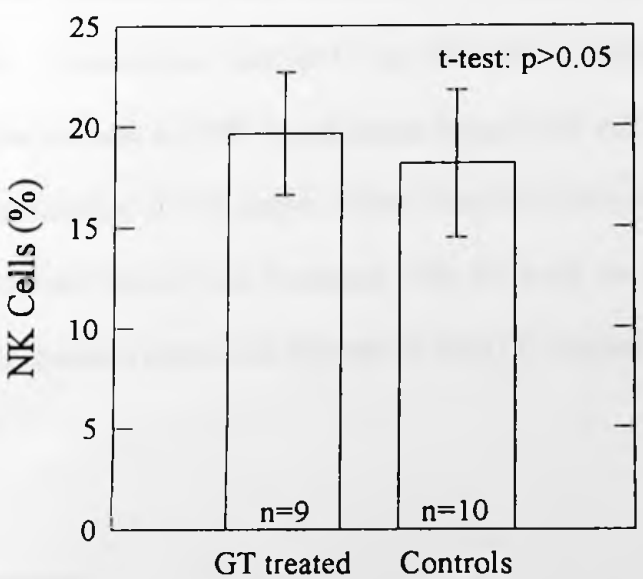


In vitro Study of Splenic NK Cells
with ConA Stimulation

Figure 27. Effect of *in vivo* GT treatment on splenic NK cells from DP/BB rats. Treatment of DP/BB rats with GT (1 μg/g body weight, intraperitoneal injection) or vehicle began at 30 days of age and continued until 65 days of age. Spleens were obtained and splenocytes were recovered. Spleen cell suspensions were preincubated for 1 hour in MEM plus 10% FCS in polystyrene flasks at 37°C in a CO_2 (5%) incubator. Splenocyte preparations were divided and half subsequently stimulated with ConA (10 μg/ml) for 1 hour. Cells were stained with FITC-conjugated anti-NK antibody and staining evaluated by flow cytometry with 10,000 cells analyzed. Columns indicate the mean % of cells staining for the NK marker, error bars show the standard error of the mean, and "n" indicates the number of rats evaluated.



In vivo Study of Splenic NK Cells



In vivo Study of Splenic NK Cells
with ConA Stimulation

PanT staining did not show a response to GT treatment *in vitro* (Figure 28) and ConA had no effect on these data. GT or vehicle was injected into DP/BB rats for the *in vivo* phase of the study. As shown by Figure 29, the results were also very similar between GT treated and control groups. ConA stimulation provided no additional effect on these data.

$CD8^+$ lymphocytes

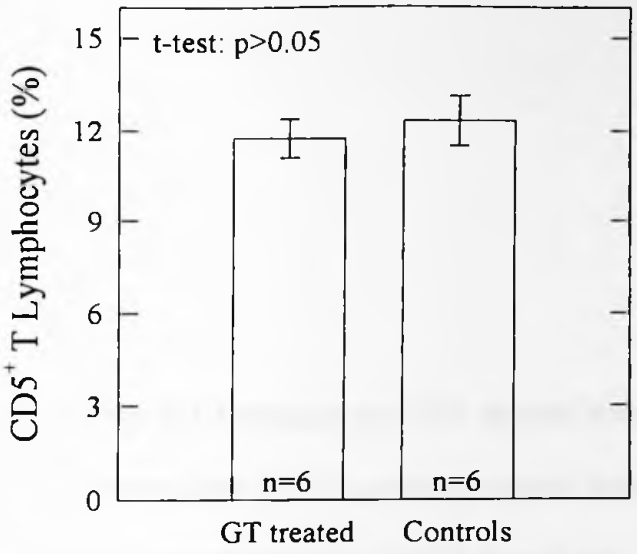
The importance of lymphocytes bearing the CD8 phenotype resides in their functions as cytotoxic effectors and also as regulatory cells. Both of these effects may be operative in IDDM and GT treatment could intervene at the level of CD8 lymphocyte abundance and function.

$CD8^+$ T splenic lymphocytes were evaluated in a method similar to that in the studies of $CD5^+$ cells. Lymphocytes incubated with GT *in vitro*, without ConA stimulation, showed a relative increase in $CD8^+$ lymphocytes (Figure 30) and this difference was enhanced by ConA stimulation to 53% above counts obtained in the absence of GT treatment. The results obtained from *in vivo* treatment with GT were not as pronounced and ConA did not serve to create a distinction between *in vivo* GT treated $CD8^+$ lymphocytes (Figure 31).

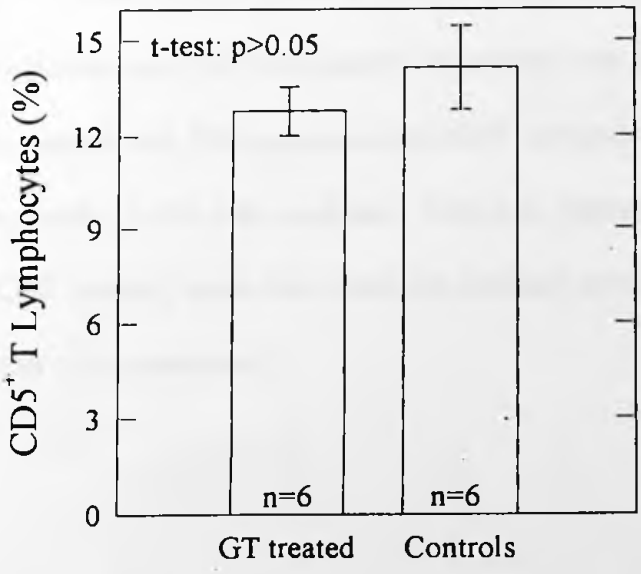
$CD4^+$ lymphocytes

$CD4^+$ T lymphocyte subsets include many different regulatory T cell types and play important roles in immune and autoimmune processes. We determined whether GT alters

Figure 28. Effect of GT on $CD5^+$ DP/BB rat splenic lymphocytes exposed to GT *in vitro*. Spleen cells from 65 day old DP/BB rats were obtained and preincubated for 1 hour at 37°C in MEM plus 10% FCS in polystyrene flasks in a CO_2 (5%) incubator. Cultures were divided into 4 aliquots, and 2 aliquots were treated with GT (1 μg/ml) for 1 hour at 37°C in a CO_2 (5%) incubator and one GT treated aliquot was subsequently incubated with ConA (10 μg/ml) for 1 hour at 37°C in a CO_2 (5%) incubator. Half of the non-GT treated control cells were also stimulated with ConA and the remainder served as unstimulated controls. Cultures were stained with PE-conjugated anti-$CD5^+$ antibody and staining evaluated by flow cytometry with 10,000 cells analyzed. Columns indicate the mean % of cells staining for the $CD5^+$ marker, error bars show the standard error of the mean, and "n" indicates the number of rats evaluated.

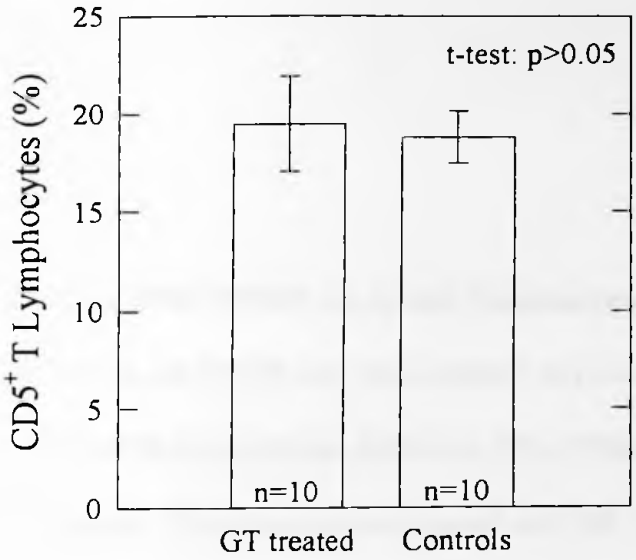


In vitro Study of PanT Splenic Lymphocytes

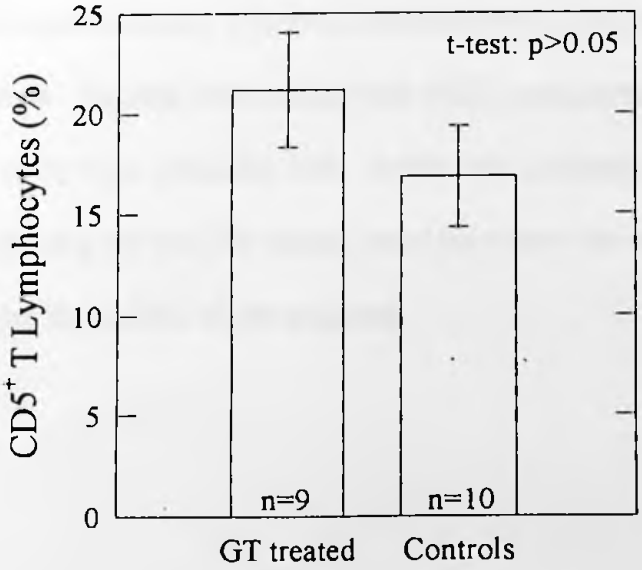


In vitro Study of PanT Splenic Lymphocytes
with ConA Stimulation

Figure 29. Effect of *in vivo* GT treatment on $CD5^+$ splenic lymphocytes from DP/BB rats. Treatment of DP/BB rats with GT (1 μg/g body weight, intraperitoneal injection) or vehicle began at 30 days of age and continued until 65 days of age. Spleens were obtained and splenocytes were recovered. Spleen cell suspensions were preincubated for 1 hour in MEM plus 10% FCS in polystyrene flasks at 37°C in a CO_2 (5%) incubator. Splenocyte preparations were divided and half subsequently stimulated with ConA (10 μg/ml) for 1 hour. Cells were stained with PE-conjugated anti-$CD5^+$ antibody and staining evaluated by flow cytometry with 10,000 cells analyzed. Columns indicate the mean % of cells staining for the $CD5^+$ marker, error bars show the standard error of the mean, and "n" indicates the number of rats evaluated.

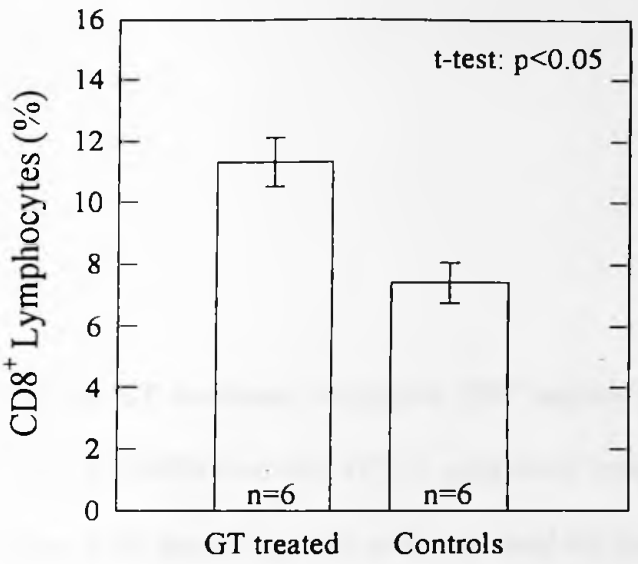


In vivo Study of PanT Splenic Lymphocytes

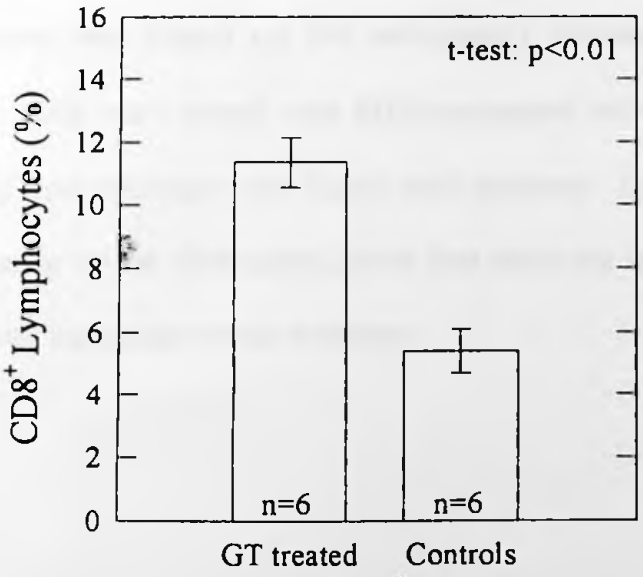


In vivo Study of PanT Splenic Lymphocytes
with ConA Stimulation

Figure 30. Effect of GT on $CD8^+$ DP/BB rat splenic lymphocytes exposed to GT *in vitro*. Spleen cells from 65 day old DP/BB rats were obtained and preincubated for 1 hour at 37°C in MEM plus 10% FCS in polystyrene flasks in a CO_2 (5%) incubator. Cultures were divided into four aliquots. Two aliquots were treated with GT (1 μg/ml) for 1 hour at 37°C in a CO_2 (5%) incubator and one GT treated aliquot was subsequently incubated with ConA (10 μg/ml) for 1 hour at 37°C in a CO_2 (5%) incubator. The other two aliquots were GT untreated controls. One was stimulated with ConA and the other served as unstimulated controls. Cultures were stained with FITC-conjugated anti-CD8 antibody and staining evaluated by flow cytometry with 10,000 cells analyzed. Columns indicate the mean % of cells staining for the CD8 marker, error bars show the standard error of the mean, and "n" indicates the number of rats evaluated.

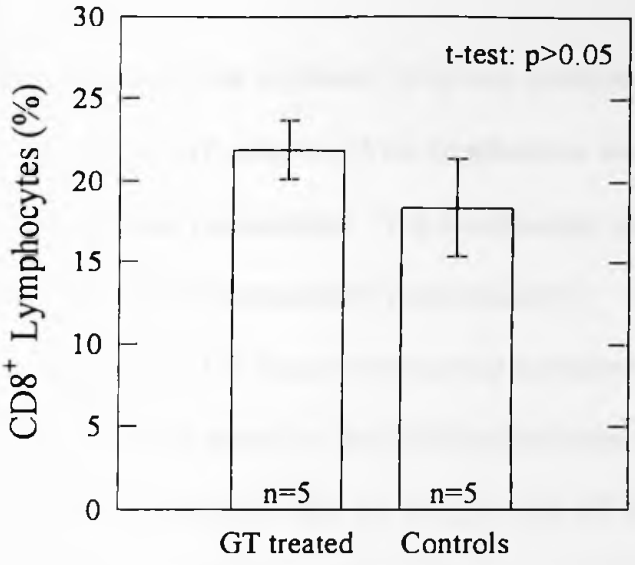


In vitro Study of $CD8^+$ Splenic Lymphocytes

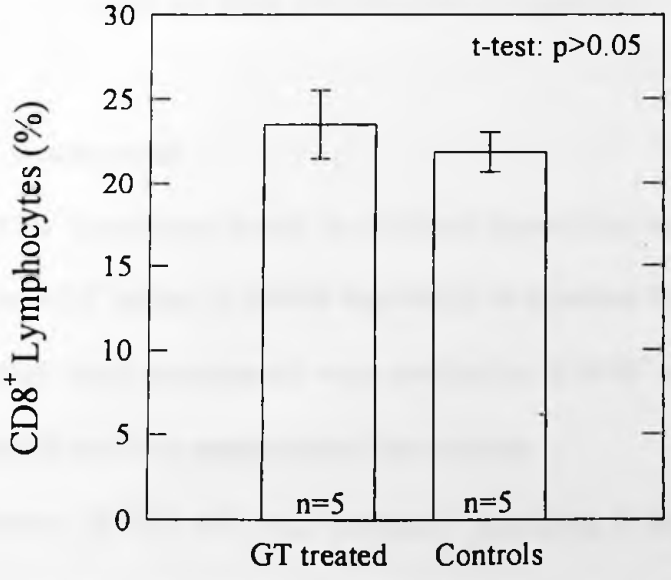


In vitro Study of $CD8^+$ Splenic Lymphocytes with ConA Stimulation

Figure 31. Effect of *in vivo* GT treatment on splenic $CD8^+$ splenic lymphocytes from DP/BB rats. Treatment of DP/BB rats with GT (1 μg/g body weight, intraperitoneal injection) or vehicle began at 30 days of age and continued until 65 days of age. Spleens were obtained and splenocytes recovered. Spleen cell suspensions were preincubated for 1 hour in MEM plus 10% FCS in polystyrene flasks at 37°C in a CO_2 (5%) incubator. Splenocyte preparations were divided and half subsequently stimulated with ConA (10 μg/ml) for 1 hour. Cells were stained with FITC-conjugated anti-CD8 antibody and staining evaluated by flow cytometry with 10,000 cells analyzed. Columns indicate the mean % of cells staining for the CD8 marker, error bars show the standard error of the mean, and "n" indicates the number of rats evaluated.



In vivo Study of $CD8^+$ Splenic Lymphocytes



In vivo Study of $CD8^+$ Splenic Lymphocytes with ConA Stimulation

the relative abundance of $CD4^+$ T cells among splenic lymphocytes exposed to GT or rats treated with GT.

The *in vitro* study involved incubation of splenic lymphocyte suspensions with GT. After 1 hour of exposure to GT, aliquots of the lymphocytes were either stimulated with ConA (10μg/ml) or remained unstimulated. The lymphocytes were labeled with PE conjugated anti CD4 mAb and were measured by flow cytometry.

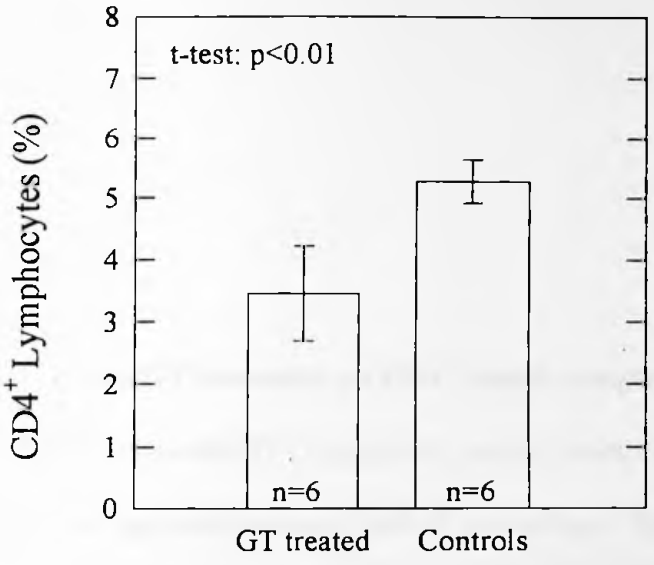
As shown in Figure 32, GT treated lymphocytes contained fewer $CD4^+$ T cells than did untreated controls. ConA enhanced the difference between GT treated lymphocytes and GT untreated controls. Control cells not treated with GT had more than twice the percentage of $CD4^+$ T lymphocytes as GT treated cell suspensions. When GT was administered chronically to DP/BB rats, no clear effect of GT on splenic $CD4^+$ populations was found and ConA did not affect this relationship (Figure 33).

$RT6^+$ lymphocytes

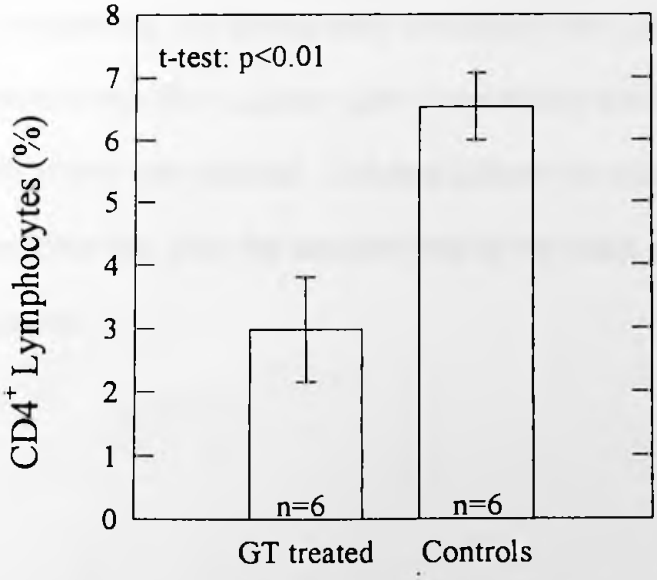
The $RT6^+$ lymphocyte subset is of critical importance to IDDM. Protocols that can restore the $RT6^+$ subset in DP/BB rats would be expected have a beneficial effect on IDDM, although most investigators view restoration of $RT6^+$ cells in DP/BB rats as a highly improbable outcome among tested interventions.

Evaluation of RT6 cells was conducted according to both *in vitro* and *in vivo* protocols as with other spleen cell phenotypic markers. As described in detail in the methods section, RT6 cell staining was measured by fluorescent intensity rather than by cell number. This technique was required by the lack of a distinct RT6 cell population that could be separated from non-staining lymphocytes. The amount of RT6 staining was sig-

Figure 32. Effect of GT on $CD4^+$ DP/BB rat splenic lymphocytes exposed to GT *in vitro*. Spleen cells from 65 day old DP/BB rats were obtained and preincubated for 1 hour at 37°C in MEM plus 10% FCS in polystyrene flasks in a CO_2 (5%) incubator. Cultures were divided into 4 aliquots, and 2 aliquots were treated with GT (1 μg/ml) for 1 hour at 37°C in a CO_2 (5%) incubator and one GT treated aliquot was subsequently incubated with ConA (10 μg/ml) for 1 hour at 37°C in a CO_2 (5%) incubator. Half of the non-GT treated control cells were also stimulated with ConA and the remainder served as unstimulated controls. Cultures were stained with PE-conjugated anti-CD4 antibody and staining evaluated by flow cytometry with 10,000 cells analyzed. Columns indicate the mean % of cells staining for the CD4 marker, error bars show the standard error of the mean, and "n" indicates the number of rats evaluated.

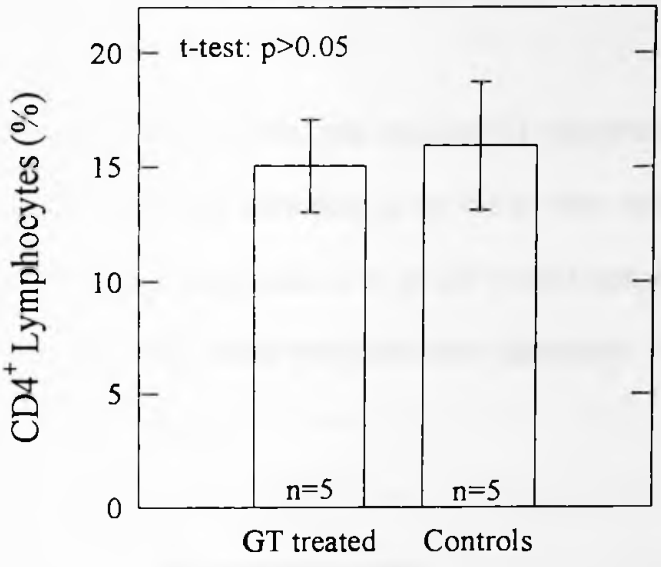


In vitro Study of $CD4^+$ Splenic Lymphocytes

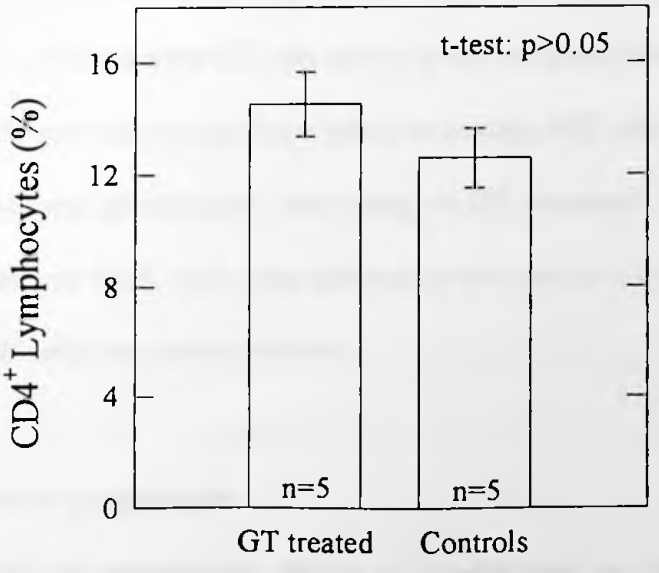


In vitro Study of $CD4^+$ Splenic Lymphocytes with ConA Stimulation

Figure 33. Effect of *in vivo* GT treatment on $CD4^+$ splenic lymphocytes from DP/BB rats. Treatment of DP/BB rats with GT (1 μg/g body weight, intraperitoneal injection) or vehicle began at 30 days of age and continued until 65 days of age. Spleens were obtained and splenocytes were recovered. Spleen cell suspensions were preincubated for 1 hour in MEM plus 10% FCS in polystyrene flasks at 37°C in a CO_2 (5%) incubator. Splenocyte preparations were divided and half subsequently stimulated with ConA (10 μg/ml) for 1 hour. Cells were stained with PE-conjugated anti-CD4 antibody and staining evaluated by flow cytometry with 10,000 cells analyzed. Columns indicate the mean % of cells staining for the CD4 marker, error bars show the standard error of the mean, and "n" indicates the number of rats evaluated.



In vivo Study of $CD4^+$ Splenic Lymphocytes



In vivo Study of $CD4^+$ Splenic Lymphocytes with ConA Stimulation

nificantly increased among cell suspensions treated with GT (Figure 34) and while ConA did not enhance this difference, the difference remained significant for ConA treated cell suspensions.

For the study of RT6 *in vivo*, rats received GT injections or control injections as described previously. Analyses were done as for the *in vitro* study. As shown in Figure 35, the mean RT6 staining index was 1.156 in GT treated samples and 0.856 in GT untreated controls, a difference which was statistically significant. ConA treatment did not eliminate this significant effect.

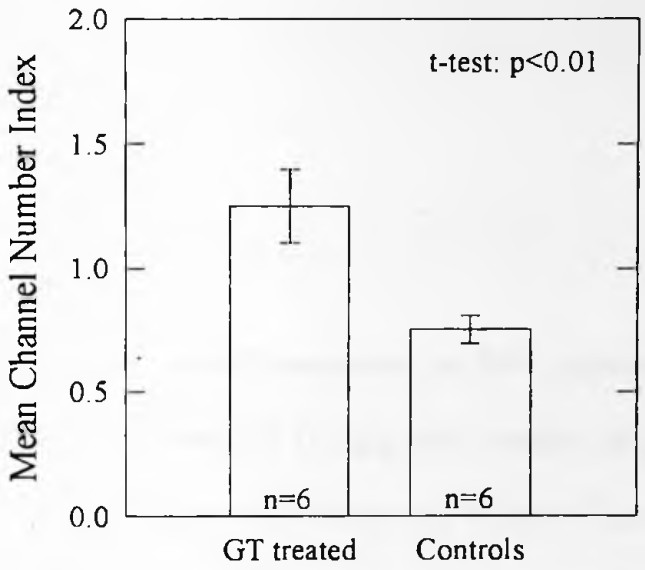
Summary of GT effect on spleen cells

Several different lymphocyte phenotypes were analyzed by flow cytometry with a number of significant findings among cells treated *in vitro* (Table 6). Fewer phenotypes were detectably affected when GT was used to treat the intact animals (Table 6). MHC II data suggest GT may have a significant effect on certain APC cells. The effector NK cells did not seem to bear an important relationship to GT treatment. However, the critically important regulatory $RT6^+$ cells were affected *in vivo* and *in vitro* and the relationship of $CD4^+$ and $CD8^+$ cells was altered *in vitro*.

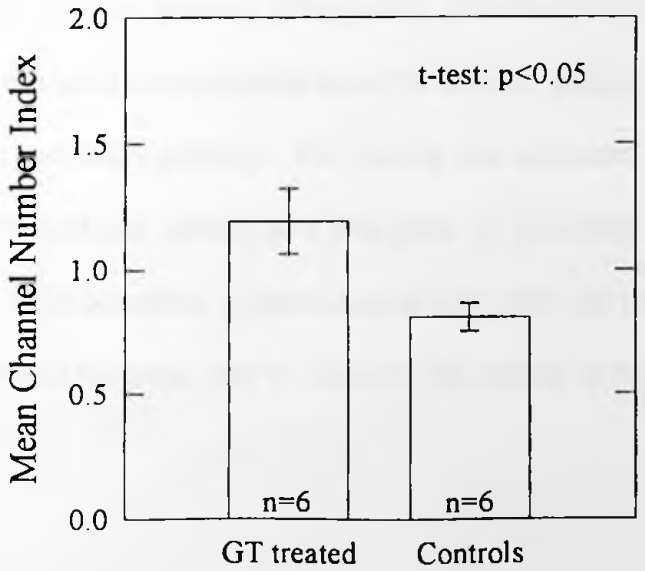
Pancreatic lymphocytes

Although the immunologic effects of diabetes may be reflected by all peripheral sites, the actual damage occurs in the pancreas. We attempted to identify effects of GT treatment on pancreatic lymphocytes through immunohistochemical staining. The results were uniformly negative (data not shown), which may have been the result of using pre-

Figure 34. Effect of GT on RT6 expression in DP/BB rat splenic lymphocytes. Spleen cells from 65 day old DP/BB rats were obtained and preincubated for 1 hour at 37°C in MEM plus 10% FCS in polystyrene flasks in a CO_2 (5%) incubator. Cultures were divided into 4 aliquots, and 2 aliquots were treated with GT (1 μg/ml) for 1 hour at 37°C in a CO_2 (5%) incubator and one GT treated aliquot was subsequently incubated with ConA (10 μg/ml) for 1 hour at 37°C in a CO_2 (5%) incubator. Half of the non-GT treated control cells were also stimulated with ConA and the remainder served as unstimulated controls. Cultures were stained with unlabeled anti-RT6 primary antibody, then FITC-conjugated anti-mouse IgG secondary antibody. The staining was evaluated by flow cytometry with 10,000 cells analyzed and plotted as a histogram as described in the methods section. Histogram data were converted to mean channel index shown by the bars. Error bars show the standard error of the mean; and "n" indicates the number of rats evaluated.

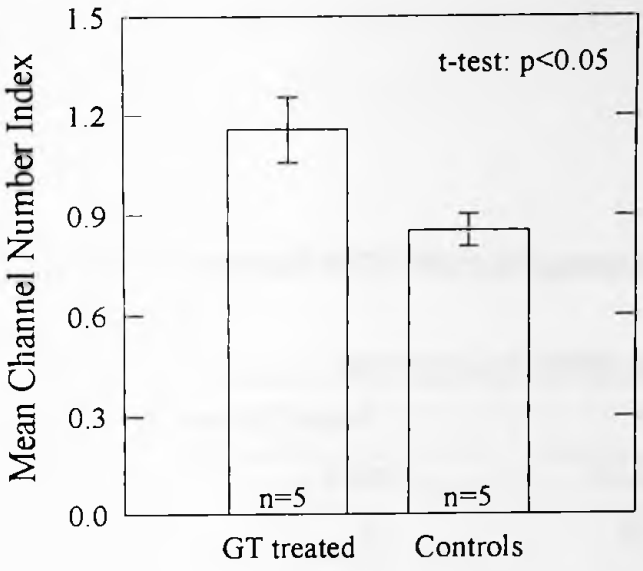


RT6$^+$ T Cell Staining of *in vitro* Study

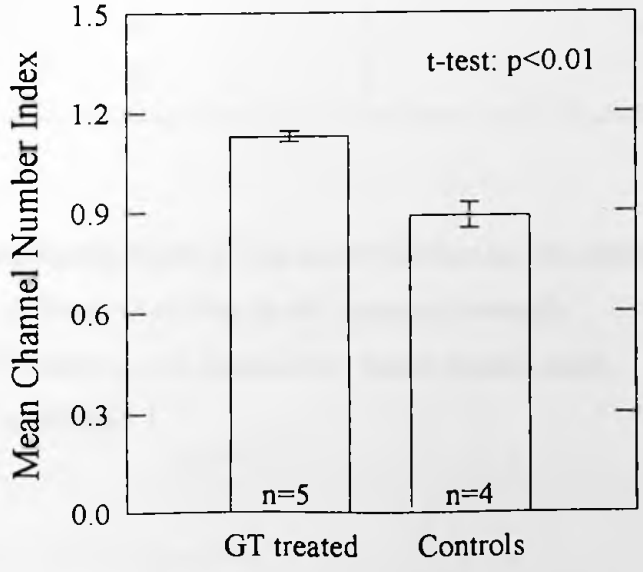


RT6$^+$ T Cell Staining of *in vitro* Study
with ConA Stimulation

Figure 35. Effect of *in vivo* GT treatment on RT6 expression from DP/BB rats. Treatment of DP/BB rats with GT (1 μg/g body weight, intraperitoneal injection) or vehicle began at 30 days of age and continued until 65 days of age. Spleens were obtained and splenocytes were recovered. Spleen cell suspensions were preincubated for 1 hour in MEM plus 10% FCS in polystyrene flasks at 37°C in a CO_2 (5%) incubator. Splenocyte preparations were divided and half subsequently stimulated with ConA (10 μg/ml) for 1 hour. Cells were stained with unlabeled anti-RT6 primary antibody, then FITC-conjugated anti-mouse IgG secondary antibody. The staining was evaluated by flow cytometry with 10,000 cells analyzed and plotted as a histogram as described in the methods section. Histogram data were converted to mean channel index show by the bars. Error bars show the standard error of the mean, and "n" indicates the number of rats evaluated.



RT6$^+$ T Cell Staining of *in vivo* Study



RT6$^+$ T Cell Staining of *in vivo* Study
with ConA Stimulation

Table 6: Summary of GT effects on spleen cells

Spleen cell population	Splenocytes of DP/BB rats			
	In vitro GT treated		*In vivo* GT treated	
	ConA -	ConA +	ConA -	ConA +
MHC II$^+$	↓	↓	NA	NA
NK	↑	↔	↔	↔
CD5$^+$	↔	↔	↔	↔
CD8$^+$	↑	↑	↔	↔
CD4$^+$	↓	↓	↔	↔
RT6$^+$†	↑	↑	↑	↑

* The data is significantly higher (↑) or lower (↓) than its GT untreated controls, or is not different (↔) from its GT untreated controls.

† Intensity of RT6 staining was measured by mean channel index.

NA: Data is not available.

diabetic animals in which lymphocytic infiltration was at a minimum. We were confident that the methods used for immunohistochemistry were not faulty since we could identify $CD8^+$ and $RT6^-$ cells in spleens of normal animals.

Mechanism of Gliotoxin in Immunomodulation

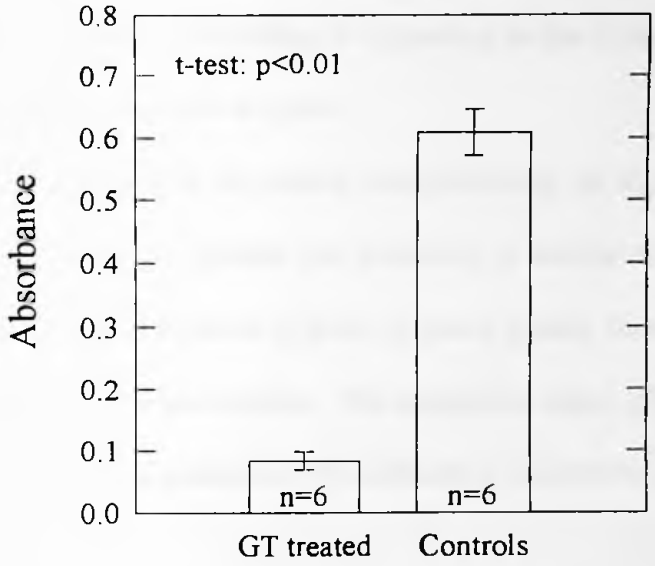
Proliferation analysis

The immune response is influenced by the opposing activities of proliferation and apoptosis. GT could exert its effects on IDDM by causing proliferation of beneficial cells such as cell subsets that suppress the autoimmune reaction. We studied the effect of GT on proliferation of DP/BB rat splenic lymphocytes with and without additional stimulation by ConA. Alamar blue was to used measure metabolic activity and to indirectly reveal cell proliferation *in vitro*.

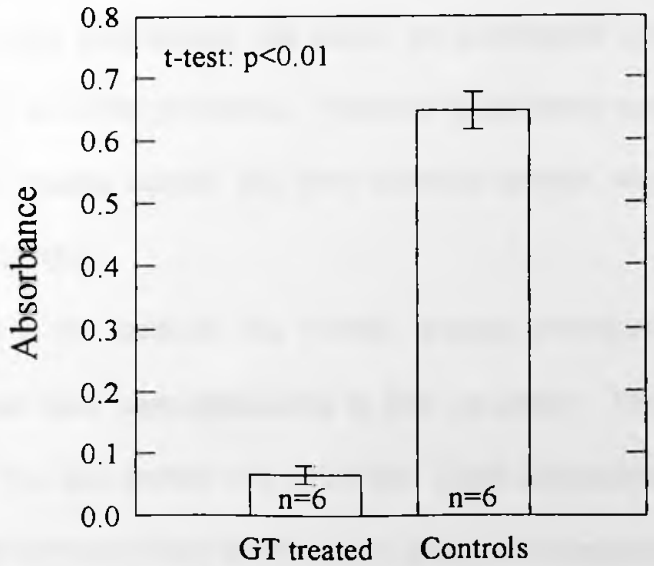
As in earlier experiments, for the *in vitro* arm of the studies, lymphocytes were isolated from spleens of 65 day old, nondiabetic DP/BB rats. The lymphocytes were divided into 2 aliquots and incubated with GT or vehicle (ethanol) in tissue culture flasks. Samples were further divided and stimulated with ConA (10 μg/ml) or were not treated with ConA. As shown in Figure 36, GT reduced leukocyte metabolic activity to a very low level even with ConA addition, suggesting that the effect of GT on IDDM probably did not result from clonal expansion.

Although GT decreased metabolic activity of ConA treated cells, we calculated a stimulation index to determine if the level of ConA stimulation was greater or smaller in GT treated cell suspensions. The mean stimulation index was 1.07 in untreated controls meaning that ConA produced 7% greater Alamar Blue reducing activity above that seen

Figure 36. Effect of GT on metabolism of DP/BB rat splenic lymphocytes exposed to GT *in vitro*. Spleen cells from 65 day old DP/BB rats were obtained and preincubated for 1 hour at 37°C in MEM plus 10% FCS in polystyrene flasks in a CO_2 (5%) incubator. Cultures were divided into four aliquots. The first aliquot of cells (10^5 cells per well) was incubated in triplicate in a 96-well microtiter plate with GT (1 μg/ml). The second aliquot was incubated without GT. The third aliquot was incubated with both GT and ConA (10 μg/ml). The fourth aliquot was incubated without GT but with ConA. The redox indicating dye, Alamar Blue was added to each well. The color changes of Alamar Blue were measured by a microplate reader at 590 nm after 72 hours at 37°C in a CO_2 (5%) incubator. The columns indicate the mean value of absorbance for the metabolic activities; error bars show the standard error of the mean; and "n" indicates the number of rats evaluated.



In vitro Metabolism Study of Splenic Leukocytes



In vitro Metabolism Study of
Splenic Leukocytes with ConA Stimulation

without ConA addition (Figure 37). GT treated lymphocytes produced a stimulation index of 0.8 indicating ConA treated cells exposed to GT showed less metabolic activity than when ConA was absent. This finding is interesting in that it suggests that GT treated cells may have lost responsiveness to ConA.

When the *in vivo* arm of the studies was performed, no significant effects on the proliferation of cells from GT treated rats compared to vehicle treated rats was found (Figure 38). With ConA stimulation (Figure 38, lower panel), mean absorbances of GT treated samples and controls were similar. The stimulation index (Figure 39) for this data did not reveal a significant augmentation of proliferative responses due to ConA.

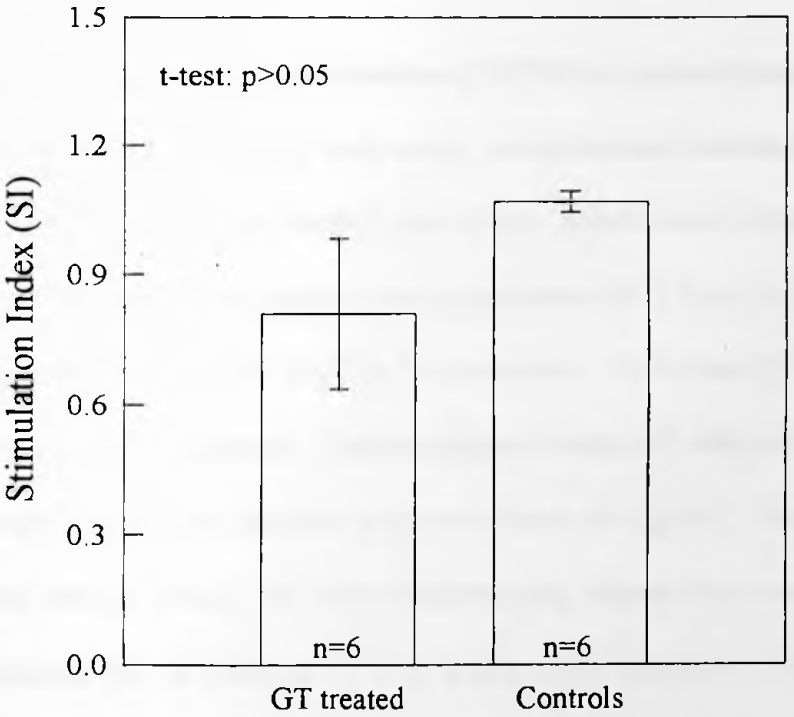
Apoptosis analysis

Because one of the key mechanisms of immune system control is selective elimination of cells through programmed cell death, we investigated apoptosis as a possible mechanism of GT in IDDM prevention. The level of apoptosis was studied both in cells derived from GT treated animals and from untreated animals whose spleen cells were treated with GT *in vitro*.

As noted in the methods, the TUNEL staining procedure was used to detect apoptotic cells and these were enumerated by flow cytometry. The apoptosis of DP/BB rat lymphocytes was also studied with or without ConA stimulation since apoptosis may be enhanced or suppressed when superimposed on cytokine production, receptor expression and T cell proliferation.

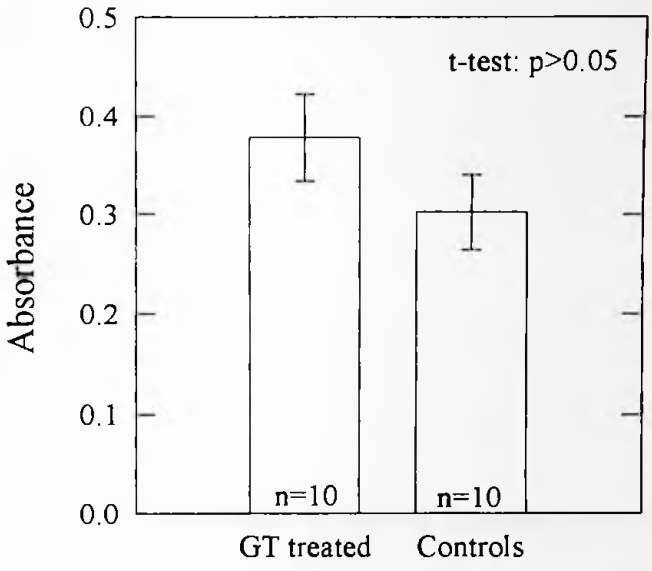
Apoptosis was measured in spleen cells from DP/BB rats treated *in vitro* with GT compared to untreated cells. As shown in Figure 40 (upper panel), GT treated DP/BB rat

Figure 37. Effect of GT on proliferation of *in vitro* GT treated DP/BB rat splenic lymphocytes. From the data presented in Figure 36, a stimulation index was calculated to quantify the influence of ConA on the splenic lymphocytes. The stimulation index is Alamar Blue reduction (A_{590}) with ConA divided by Alamar Blue reduction without ConA. The columns indicate the mean value of proliferation; error bars show the standard error of the mean; and "n" indicates the number of rats evaluated.

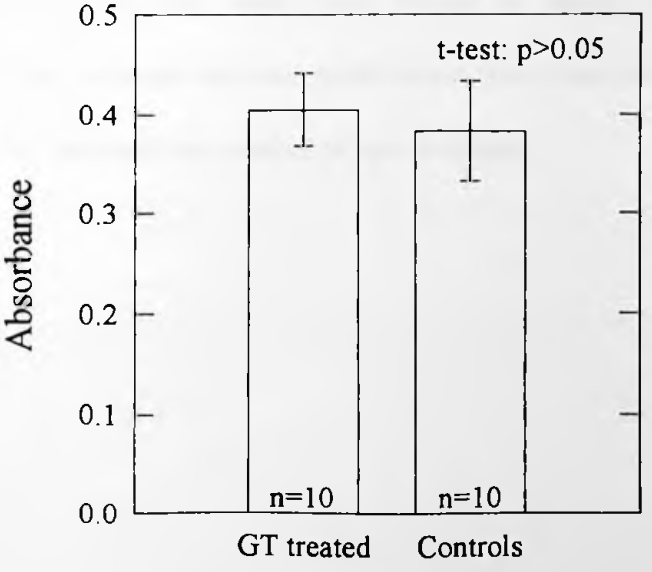


In vitro Proliferation Study of Splenic Leukocytes

Figure 38. Effect of GT on metabolism of DP/BB rat splenic lymphocytes. Treatment of DP/BB rats with GT (1 μg/g body weight, intraperitoneal injection) or vehicle began at 30 days of age and continued until 65 days of age. Spleens were obtained and splenocytes recovered. Spleen cell suspensions were preincubated for 1 hour in MEM plus 10% FCS in polystyrene flasks at 37°C in a CO_2 (5%) incubator. Cells from GT or control rats were each divided into two aliquots. The first aliquot of cells (10^5 cells per well) was incubated in triplicate in a 96-well microtiter plate with ConA (10 μg/ml). The second aliquot was incubated without ConA. The redox indicating dye, Alamar Blue was added to each well and incubated for 72 hours at 37°C in a CO_2 (5%) incubator. The color changes of Alamar Blue were measured by a microplate reader at 590 nm. The columns indicate the mean value of absorbance for the metabolic activities; error bars show the standard error of the mean; and "n" indicates the number of rats evaluated.

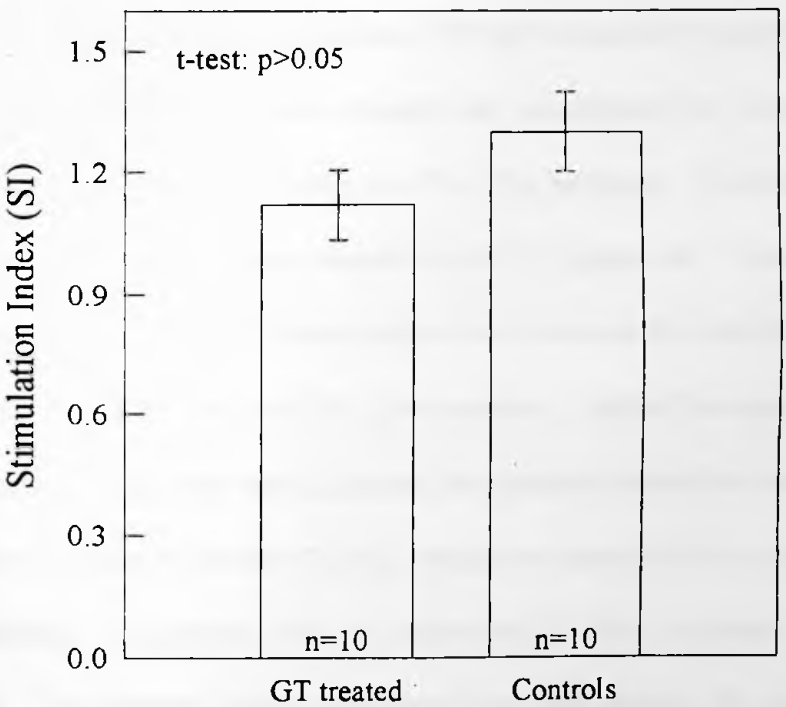


In vivo Metabolism Study of Splenic Leukocytes



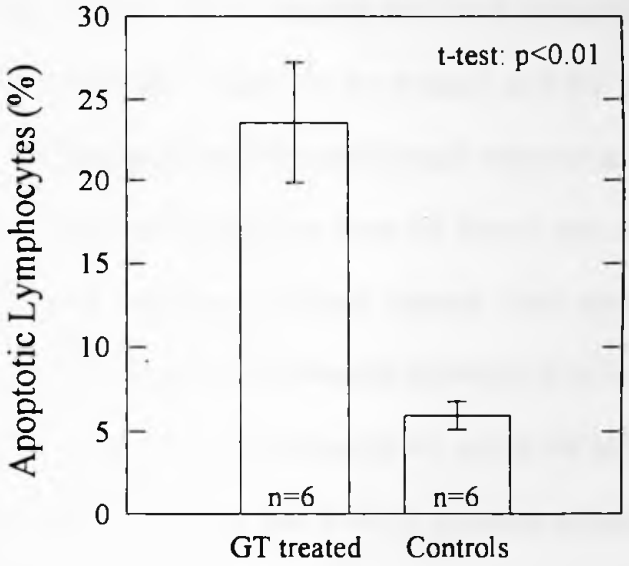
In vivo Metabolism Study of
Splenic Leukocytes with ConA Stimulation

Figure 39. Effect on proliferation of splenic lymphocytes from *in vivo* GT treated DP/BB rat. From the data presented in Figure 38, a stimulation index was calculated to quantify the influence of ConA on the splenic lymphocytes. The stimulation index is Alamar Blue reduction (A_{590}) with ConA divided by Alamar Blue reduction without ConA. The columns indicate the mean proliferation; error bars show the standard error of the mean; and "n" indicates the number of rats evaluated.

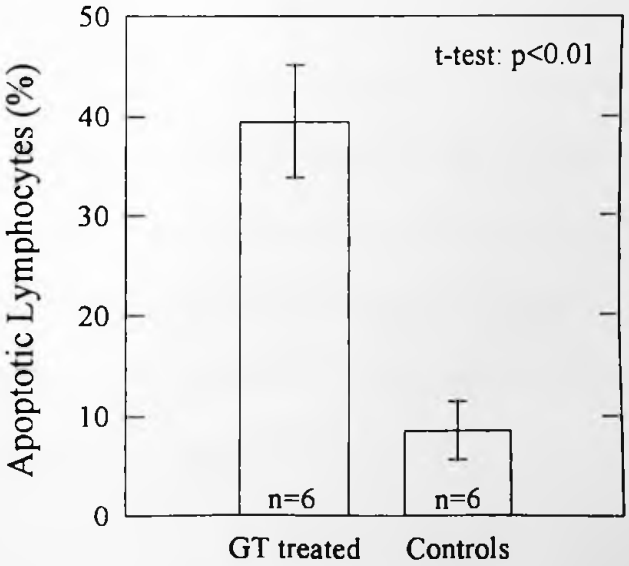


In vivo Proliferation Study of Splenic Leukocytes

Figure 40. Effect of GT on apoptosis of DP/BB rat splenic lymphocytes. Spleen cells from 65 day old DP/BB rats were obtained and preincubated for 1 hour at 37°C in MEM plus 10% FCS in polystyrene flasks in a CO_2 (5%) incubator. Cultures were divided into 4 aliquots, and 2 aliquots were treated with GT (1 μg/ml) for 1 hour at 37°C in a CO_2 (5%) incubator and one GT treated aliquot was subsequently incubated with ConA (10 μg/ml) for 1 hour at 37°C in a CO_2 (5%) incubator. Half of the non-GT treated control cells were also stimulated with ConA and the remainder served as unstimulated controls. Cultures were stained with the TUNEL method as described in the methods section. The FITC staining of apoptotic cells was determined by flow cytometry with 10,000 cells analyzed. The columns indicate the mean % of cells staining for apoptosis; error bars show the standard error of the mean; and "n" indicates the number of rats evaluated.



In vitro Apoptosis Study of Splenic Lymphocytes

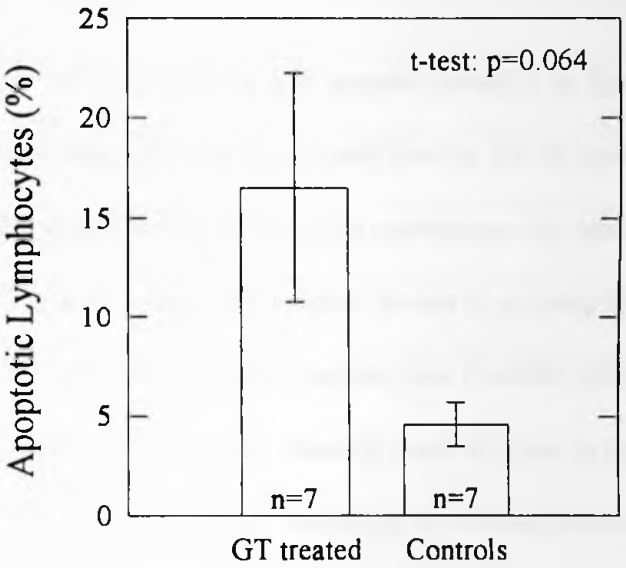


In vitro Apoptosis Study of
Splenic Lymphocytes with ConA Stimulation

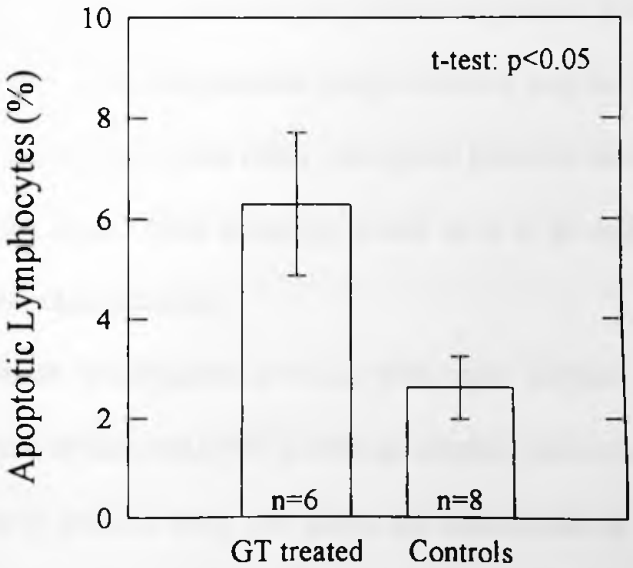
splenic lymphocytes showed 23.6% apoptosis, a level significantly higher than the 6.0% seen in untreated controls. When repeated with ConA stimulation, this same experiment showed a four-fold difference (Figure 40, lower panel) as it did without ConA ($P<0.01$).

In vivo GT treatment provided results which were not as pronounced as for *in vitro* GT treatment. Splenic lymphocytes from GT treated rats showed 15.67% apoptotic cells while 7.50% of cells from untreated controls were apoptotic (Figure 41, upper panel), and ConA stimulation of lymphocytes recovered from GT treated or control rats showed a significant difference in apoptosis levels, with 6.3% of splenic lymphocytes from GT treated DP/BB rats apoptotic and 2.6% of untreated controls apoptotic (Figure 41, lower panel).

Figure 41. Apoptosis of splenic lymphocytes from *in vivo* GT treated or control DP/BB rats. Treatment of DP/BB rats with GT (1 μg/g body weight, intraperitoneal injection) or vehicle began at 30 days of age and continued until 65 days of age. Spleens were obtained and splenocytes were recovered. Spleen cell suspensions were preincubated for 1 hour in MEM plus 10% FCS in polystyrene flasks at 37°C in a CO_2 (5%) incubator. Splenocyte preparations were divided and half subsequently stimulated with ConA (10 μg/ml) for 1 hour. Cells were stained with the TUNEL method as described in the method section to determine the apoptotic cell percentage in splenic lymphocytes. The FITC staining of apoptotic cells was evaluated by flow cytometry with 10,000 cells analyzed. The columns indicate the mean % of cells staining for the apoptosis; error bars show the standard error of the mean; and "n" indicates the number of rats evaluated.



In vivo Apoptosis Study of Splenic Lymphocytes



In vivo Apoptosis Study of
Splenic Lymphocytes with ConA Stimulation

DISCUSSION

Although various methods and reagents intended to lessen the effects of IDDM have been studied, none of them has proved ideal in IDDM prevention and/or treatment. The research presented in this dissertation summarizes our efforts to evaluate an immunomodulator, GT, as a potential preventive for use in averting IDDM development. Previous work in our laboratory showed that the yeast *Candida albicans* produces GT which may inhibit host immune responses, allowing yeast infection in the human vaginal tract to persist without immune elimination. Although our current understanding of GT as an immune response modifier is limited to its effects on PMN's, the discovery of its action on white blood cells led us to hypothesize that GT may prevent IDDM in DP/BB rats. We also hypothesized that GT exerts selective immunosuppressive activities which may distinguish it from other immunosuppressive fungal products, such as CsA. GT may have specific effects in certain cell types rather than global functions directed toward all immune cell types as CsA does. Such selectivity would serve as an important innovation in immunosuppressive chemotherapy.

The present investigation provided three major findings. First we demonstrated that GT treatment of diabetes prone animals successfully delayed onset of IDDM. To our knowledge, this is the first study that shows the effectiveness of GT in interrupting autoimmune pathogenesis. Further emphasizing its effectiveness, we found that GT treatment decreased blood glucose levels in pre-diabetic DP/BB rats. Second, GT selectively influences several regulatory T cell subsets without causing the same reaction among all lymphocyte subpopulations, which is important because the clinical promise of immune

modulator therapy requires selective rather than universal immunosuppression. Third, GT induces lymphocyte apoptosis which provides an explanation of how GT may exert its effect on the immune system. These three findings, coupled with the demonstration that GT has limited systemic toxicity in the animal model studied, provides important insights into GT and its immunomodulating function in relation to autoimmunity inhibition, and further indicates that GT could have future potential in human disease. This new knowledge may help provide better understanding of IDDM both scientifically and clinically.

Limited systemic toxicity of GT

Before undertaking studies of diabetes prevention it was necessary to evaluate GT toxicity. It was not the intention of this project to perform a complete toxicologic investigation, but rather we limited our study to experiments that would suffice to allow preliminary animal studies. We first exposed an insulin-secreting β cell line, RIN 38 insulinoma cells, to GT at various concentrations and determined the viability and insulin secreting ability of these cells following GT challenge. GT treatment did not alter the viability of RIN 38 cells at concentrations of 1 μg/ml or less and while some effect (28% reduction) on insulin secretion was seen at 1 μg/ml GT concentration, insulin secretion was not seriously compromised. We also noted that the rate of body weight gain during GT treatment of DP/BB rats (from age 30 days to 65 days) was normal. Leukocyte counts after 10 GT injections over a 24 day period failed to elicit leukopenia. Finally, liver and kidney histologic sections of GT treated animals were essentially indistinguishable from those of control DP/BB rats which had not been exposed to chronic GT treatment. These results gave us confidence that GT at a dosage of 1 μg GT per gram body weight is not too toxic

for use in these experimental animals. In fact, when we treated DP/BB rats with GT at this dosage, GT showed a truly salutary biologic effect in terms of diabetes prevention, but the experimental animals remained overtly healthy during prolonged observation.

Delayed onset of IDDM by GT

When GT treatment of DP/BB rats was started at 30 days of age and continued to 120 days of age, GT treated animals showed an average of diabetes onset of 98 days compared to 82 days average age of onset among the control rats. This finding coupled with the incidence of 90% diabetes within 120 days among control rats versus 55% incidence among GT-treated animals proved significant by life-table analysis. Although the GT treatment study demonstrated that GT is able to influence the development of autoimmunity, to achieve its beneficial effects the GT treatment had to start at 30 days of age. GT treatment begun at 40 days was not effective in diabetes prevention. The reason GT treatment is effective when started at 30 days of age but not at 40 days of age may relate to the existence of different phases of autoimmune pathogenesis. In autoimmunity one can envision two stages, an early stage in which autoimmune cells are generated and a late stage in which autoimmune damage occurs, driven by effector cells such as macrophages, NK cells or CTLs. The finding that the intervention is effective at 30 days, but not 40 days, suggests that GT may be influencing the early stages of autoimmunity rather than the late, effector phase. This issue will be discussed again in greater detail when the cell types affected by GT are considered. Interestingly, our findings are consistent with other studies in that several investigators have identified a window of intervention opportunity which may relate to some critical milestone in lymphatic system maturation. Gottlieb and Rossini

(1993) called this phenomenon the "window of vulnerability" during which time immune interventions to prevent IDDM in DP/BB rats may be effective. In this window period, autoimmune processes are not fully established and effector cells are minimally active allowing immune interventions to change the course of IDDM. When the window is closed, the effector phase is fully active and the immunoregulatory phase is matured so that most immune interventions are no longer effective.

Complementing its effect on disease incidence, we also found that GT treatment significantly reduced blood glucose levels among both diabetic and prediabetic rats. The reason why GT lowered blood glucose levels was not directly investigated as part of this study, but the observation is consistent with a preservation of β cell function. Serum glucose is controlled by various metabolic hormonal factors, but primarily through reciprocal interaction of insulin and glucagon. Insulin is produced by the β cells of the islets of Langerhans in the pancreas. The signals for the release of insulin from the β cells are a high blood sugar level and, to a lesser extent, high plasma levels of some amino acids. The release of insulin in response to a glucose load shows two phases, an early burst of insulin release and a later slow output of hormone. Insulin reduces cAMP levels, probably by speeding up the destruction of cAMP by phosphodiesterase. Insulin suppresses gluconeogenesis and at the same time increases glycolysis. Glucagon has activity that opposes insulin and can increase gluconeogenesis by increasing synthesis of several enzymes (Stryer, 1988). The diabetic patient is characterized by a deficiency of pancreatic islet β-cells resulting in loss of adequate of insulin production. Glycolysis is inhibited and the blood glucose level increases. When the glucose level reaches a critical point, the patient

becomes diabetic and the toxicity of glucose accumulation will lead to damage of various organs.

There are several possible explanations for reduced glucose levels among GT treated rats. GT could increase insulin secretion, as occurs with the oral hypoglycemic sulfonylureas, tolbutamide and tolazamide, resulting in lower blood glucose levels. This is unlikely since studies of GT action on RIN 38 insulin secretion showed that GT did not increase, and actually reduced somewhat, insulin production by this β cell line. Blood glucose could be controlled in GT treated rats because of direct protection of β cells from autoimmune attack which would preserve insulin producing capacity. However this is not consistent with our early study in which GT treatment started after 40 days of age is not protective, suggesting that GT is not able to influence the effector phase of autoimmunity. GT could also lower blood glucose through β cell regeneration. This possibility was not supported by the RIN 38 cell studies. Another explanation for the blood glucose effects is decreased peripheral insulin resistance. Since peripheral insulin resistance is not the main mechanism of IDDM in DP/BB rats, this is unlikely to be the role of GT. The elimination of these various explanations returns our focus to immunologic mechanisms. Many other studies (Bowman, Leiter and Atkinson, 1994) on IDDM prevention using a variety of approaches also emphasizes the immunopathogenic mechanism, as well. Therefore it is likely that GT decreases blood glucose by preservation of β cell function by preventing or modifying autoimmune disease.

Selective influences of GT on regulatory T cell subsets

Published studies support GT function as an immune suppressor (Sutton, Waring and Mullbacher, 1996; Murayama, et al 1996; Sutton, et al., 1996) which has led to its use in prevention of transplant rejection (Sutton, et al., 1995; McMinn, Halliday and Muller, 1990; Mullbacher, et al., 1988; Lissing, Tuch and Suranyi, 1988). Early work from our laboratory showed that GT suppressed PMN oxidative burst in response to stimulation by complement components (Shah, et al. 1995). In contrast, the results from the present study demonstrated that GT induced lymphocyte apoptosis, suppressed lymphocyte proliferation and altered the proportions of certain lymphocyte subsets. Both the lower blood glucose levels in GT treated animals and the delay in diabetogenesis are all likely attributable to the effects of GT on the immune system of DP/BB rats.

A key issue raised by the protective effect of GT is what parts of the immune system is/are affected. One drawback of many immune interventions, such as CsA, is their fairly global effects on the immune system. For various reasons, a new immunotherapy reagent should possess a more specific function, preferably one which will prevent autoimmune disease without causing increased susceptibility to infection or cancer in drug recipients.

The research approach we pursued was to investigate whether GT had a demonstrable effect on lymphocyte cell subsets in experimental animals prone to diabetes. These analyses were conducted both on isolated cells treated with GT and on animals treated with GT. Although study of GT's effects on isolated cells is more sensitive, investigation of GT's effects *in vivo* is essential because that is the ultimate place where GT could be valuable, and where we demonstrated its efficacy in diabetes prevention.

As noted earlier, diabetes preventive measures may interfere with various stages of disease development. Thus, we investigated the MHC II cells as potential indicators of antigen presentation, NK cells as indicators of cytotoxic effects, and $CD4^+$, $CD8^+$ and $RT6^+$ T cells as indicators of an immune regulatory component of the anti-diabetic effect. Such results must be interpreted with caution because broad categories of lymphocytes do not completely and uniquely correlate with function. For example, NK cells function primarily as effectors but can also be involved in regulatory functions. CD8+ T cells can be effectors but also regulators. Macrophages and dendritic cells are mainly APC's, but B cells can function as APC cells as well.

For *in vitro* studies, splenic cells were isolated from 65 day old prediabetic DP/BB rats then treated with GT. For *in vivo* studies, splenic cells were also isolated from prediabetic animals at 65 days of age, however these animals were treated with GT from 30 days of age to 65 days of age. The reason prediabetic DP/BB rats were chosen for this study was because GT was ineffective late in pathogenesis and we would envision immune intervention occurring during the earlier prediabetic period versus later. The splenic cells were studied under either with ConA or without ConA stimulation. ConA is the mitogen to induce T cell proliferation and to enhance cytokine production and cytokine receptor expression. Proliferating cells are more susceptible to drug treatment so that the effects of GT to T cell subsets may be measured more sensitively.

One of the prevailing theories of autoimmune pathogenesis implicates faulty antigen presentation in development of the disease process. For this reason we investigated GT's effect on MHC II cells which includes APC'S. Faulty antigen presentation may display self antigens as foreign and induce autoimmunity. Antigen presentation may also

stimulate regulatory T cell activation and enhance autoimmune pathogenesis. The activated regulatory T cells may also increase the activity and/or number of APC to further enhance the autoimmune antigen presentation and the autoimmune process. Under these conditions, more cells bearing MHC class II surface markers could be expected. Our efforts provide limited information in this area because our cell preparation methods were not designed to maximize macrophage recovery and even if a very robust yield of macrophage and dendritic cells had been achieved, the antigen presenting activity would need to be evaluated in a specific manner separate from enumeration of MHC II^+ cells. We did not initially observe differences in MHC II staining among GT-treated splenic cells compared to non-treated cells in which lymphocytic cells were gated. This population would have been expected to include B lymphocytes. Although B cells may present antigens, they are probably of little consequence in the antigen presentation pertaining to IDDM. Thus it was not surprising that MHC II staining was not affected in this evaluation. To provide a more comprehensive evaluation, we re-gated and re-analyzed the MHC II data to include larger cells and those with potentially greater granularity. The cells in this gate were highly sensitive to GT, although we cannot definitively state the identity of these cells. Because this analysis showed that at least some MHC II positive cells were eliminated by GT we cannot preclude a possible effect of GT on APCs. Although GT may or may not have an effect on APCs, we did not look for individual subsets of MHC class II positive cells. If GT treatment only affected one subset, our methods would not have been sensitive enough to detect such subtle differences. MHC II positive cells were used to investigate antigen presentation as the step in the autoimmune process were GT has its effect, and these cells were not clearly implicated.

Evaluation of NK cells was used to investigate one of the late steps in diabetogenesis, namely the effector phase, either *in vivo* or *in vitro*. Enumeration of NK cells yielded the results which is not significant different between GT treated lymphocytes and controls. This is not surprising, since the study showing that GT treatment beginning at 40 days of age was ineffective. IF GT affects IDDM pathogenesis through NK cells, GT treatment beginning after 40 days of age should also be effective This implies that the main effect of GT is earlier than the NK-dominated phase of pancreatic damage. Having demonstrated that the earliest (antigen-presenting) and latest phase (NK-dominated) of the autoimmune reactions are unlikely to be the main targets of GT action, we devoted our attention to T-cells, and in particular, the immunoregulatory aspects of the disease.

Most differences among splenic cells revealed by this study occurred in T cell populations. The change could be within different T cell subsets. The occurrence of genuine changes in T cell subsets were revealed by studies of specific surface markers, the most notable being $CD4^+$. In addition, specific subset changes were most clearly seen from *in vitro* data. Although not all effects seen in the *in vitro* studies were replicated in the *in vivo* studies, many of the observations *in vivo* were qualitatively similar to those seen in *in vitro*. As will be discussed in greater detail later, the *in vivo* studies tend to be somewhat less sensitive in depicting the effects of GT's on lymphocyte populations. This may be primarily due to pharmocokinetic and pharmacodynamic considerations. Because T cells in general are probably too broad a category to delineate GT's effects, we focused on CD4+ and CD8+ cells, each of which may play regulatory roles in normal and abnormal immune responses. $CD4^+$ T cell percentage was decreased and $CD8^+$ T cell percentage was increased in the *in vitro* experiments. Similar results were obtained *in vivo* that

statistical significance was not attained due to the small number of animals. Together, such results portray GT as a selective immunotoxin or immunomodulator, a key property for a useful immunotherapeutic drug.

Earlier studies have shown the effects of CD4 and CD8 lymphocytes in IDDM and other autoimmune diseases. Both CD4 and CD8 T cells were required for successful adoptive transfer of disease with diabetic spleen cells into young NOD mice (Bendelac, et al., 1987) and irradiated adult NOD mice (Miller, et al 1988; Barey, et al., 1991), but the relative roles of the two subsets remains controversial. CD4 T cells from donor NOD mice were critical for diabetes development in T cell-depleted NOD mice (Hanafusa, et al., 1988). Purified CD4 T cells from diabetic donor spleens transferred disease at low efficiency in immunodeficient NOD mice, although purified CD8 did not (Christianson, Shultz and Leiter, 1993). Other studies indicated that the role of CD4 T cells in IDDM pathogenesis was to recruit CD8 T cells as final effectors in islet β-cell destruction (Thivolet, et al., 1991; Matsumoto, et al., 1993). As shown by a recent study by Peterson and Haskins (1996), one $CD4^+$ T cell clone, BDC-6.9 can transfer IDDM without help from host B cells, $CD4^+$ T cells or $CD8^+$ T cells. A second $CD4^+$ T cell clone, BDG-2.5 only induced IDDM when $CD8^+$ T cells were present. Different T cell clones require different levels of accessory cell involvement. BDC-6.9 cells arise later in the disease process and can act alone. DBC-2.5 may be representative of the earliest diabetogenic CD4 T cells that require help from CD8 T cells, and would become an appropriate cell subset to investigate in future studies of the effects of GT.

Collagen-induced arthritis (CIA) is a different but useful experimental autoimmune disease elicited by immunization with type II collagen. As shown by one recent study

(Amano, et al., 1995), CIA incidence in CD8 -/- mice was significantly decreased compared to the incidence among CD8 +/- mice. This result suggests a role for $CD8^+$ T cells in initiating CIA. Interestingly, CD8 deficient mice were more susceptible to a second induction of arthritis after remission of initial disease, pointing toward an immunoregulatory role for $CD8^+$ T cells (Tada, et al., 1996). $CD8^+$ T cells have been implicated as an initial element required to recruit $CD4^+$ T cells to islets and may also function as a final effector in β cell damage.

When an individual becomes diabetic, the $CD4^+$ T cell percentage increases significantly and $CD8^+$ T cell percentage declines slightly. A human IDDM study (Ilonen, Surcel and Kaar, 1991) indicated that $CD4^+$ and $CD8^+$ T cell subsets were 38% and 27% respectively before diabetes and 44% and 26% after the patients developed overt diabetes. The ratio of CD4/CD8 T cells was also increased. Since $CD4^+$ and $CD8^+$ T cells are important regulatory lymphocytes, and in view of the human data mentioned, our observed decrease in $CD4^+$ T cell numbers, and increase in $CD8^+$ T cell numbers and a decreased CD4/CD8 T cell ratio are noteworthy as they are consistent with a beneficial effect toward IDDM. We showed that GT treatment decreased the relative number of $CD4^+$ T cells and increased the relative number of $CD8^+$ T cells, and the ratio of CD4/CD8 T cells was decreased, although the effect was limited to *in vitro* observation.

In vitro GT treatment is undoubtedly more potent than *in vivo* treatment with respect to T cell population dynamics for several reasons. The GT concentration *in vitro* remains constant and probably attains a higher local concentration. In addition, *in vitro* conditions provide a constant environment, uniform cell populations and no biological clearance mechanisms as would exist *in vivo*. *In vivo* GT treatment cannot be expected to

be as effective as *in vitro* for several additional reasons. The GT dose used in BB rats was chosen to avoid toxicity rather than maximize its biological effects. The *in vivo* dosage schedule was episodic which should result in peak and trough tissue levels of GT. This pulsatile treatment was originally intended to let animals recover somewhat after acute exposure to GT to prevent cumulative toxicity, but we had no way to judge the therapeutic index (difference between diabetes protective dose and toxic dose) in these preliminary studies. GT was injected intraperitoneally and it is unknown how much GT reaches the spleen, pancreas and other relevant sites. Indeed, the effects seen among spleen cells could have been generated in the bone marrow, but without specific studies, the ultimate source of *in vivo* effects is difficult to predict. Our study did clearly indicate that sufficient drug was present *in vivo* to prevent disease even though it may not have resulted in detectable effects on $CD4^+$ and $CD8^+$ T cells.

One of the most important advances in understanding diabetogenesis in the DP/BB rat has been the discovery of the $RT6^+$ marker on mature lymphocytes. Because of its significance in the DP/BB rat, we focused attention on this lymphocyte subset which is nearly absent in DP/BB rats and is highly associated with diabetes incidence. RT6 protein is not totally absent from DP/BB animals and is detected at a very low level on a small number of cells. Explanations for the $RT6^+$ T cell subset deficiency include premature cell death and gene regulation defects. RT6 in normal rats is present on mature cells and death of these cells prior to full maturation could lead to low numbers of $RT6^+$ cells. Alternatively, failure of cells destined to become $RT6^+$ to produce adequate quantities of $RT6^+$ surface antigen could also be responsible for low levels of RT6. Although GT treatment did not

restore $RT6^+$ cells to normal levels in DP/BB rats, treatment did increase RT6 surface marker detection both *in vivo* and *in vitro*.

Several previous investigations have underscored the importance of the RT6 surface marker among DP/BB rats in contrast to DR/BB rats, WF rats and LEW rats at different ages. Neither RT6.1 nor RT6.2 was found on DP/BB rat T cells, but they were found on T cells of other rats (Greiner, et al. 1986). A soluble form of RT6 rat lymphocyte alloantigen was detected in serum of DP/BB, DR/BB and WF rats by Western blot analysis, but DP/BB rats circulated less RT6 alloantigen than did DR/BB or WF rats (Waite, et al. 1993). Anti RT6.1 mAb injection into DR/BB rats depleted >95% of peripheral RT6 T cells, but did not reduce levels of circulating T cells or the *in vitro* response of spleen cells to mitogen. If this treatment was started at 30 days of age, it significantly enhanced the frequency of diabetes in DR/BB rats. If anti-RT6 antibody treatment was started at 60 days of age, it failed to produce these effects (Greiner, et al. 1987).

The immunoregulatory function of the $RT6^+$ T cell subset has been confirmed by many studies but the reason for the absence of RT6 surface marker in DP/BB rats was investigated by Crisa, et al (1993). They discovered that an mRNA encoding RT6 protein was present in spleen cells of diabetes-prone rats and the nucleotide sequence of this transcript revealed an intact coding region for the RT6 alloantigen. In addition, RT6 mRNA was translated *in vivo*, but the RT6 protein in DP/BB rat lymphocytes was less than 10% of the amount found on DR/BB rat lymphocytes. The intact phosphatidylinositol linkage of the molecule to the cell surface was detected in DP/BB rat lymphocytes by immunoprecipitation. These investigators concluded that defects in RT6 gene regulation or other cellular defects leading to premature cell death were operative in these animals.

Because the effect of GT on RT6 was more subtle than its effects on such cell populations as $CD4^+$ T cells, a different method of analysis was applied to RT6 data. The mean channel number is a measurement derived from flow cytometry evaluation in which the average fluorescent intensity of a cell population is recorded. $RT6^+$ T cell staining was measured by this method because a distinct population of intensely stained cells was not present in splenocytes recovered from DP/BB rats.

Crisa et al (1993) indicated that RT6 protein expressed is reduced in DP/BB rats and altered RT6 gene regulatory mechanisms may be responsible for RT6 deficiency. We detected higher anti-RT6 immunofluorescent staining in GT treated splenic lymphocytes which may result from enhanced expression of RT6 protein on the surface of lymphocytes after exposure to GT, although direct investigation of RT6 protein levels would be appropriate to verify this explanation. After gene transcription, a series of enzymes and regulatory proteins are involved in translation, modification and protein targeting. Synthesized protein will fail to be expressed on membranes if any of these enzyme and regulatory proteins are non-functional. GT may bind the defective enzyme or regulatory protein to improve its function, leading to greater RT6 protein expression. Known characteristics of GT support the possible interaction with biologically active proteins. GT is a small hydrophobic molecule which passes through cell membranes. The disulfide group of GT can bind similar groups on regulatory proteins to modify their regulatory functions. Although $RT6^+$ T cells were not restored to levels comparable to those seen in DR/BB and other normal rats, more RT6 protein on the lymphocyte surface was observed which may increase immunoregulatory function which in turn results in decreased β cell loss.

We can not exclude the possibility that GT prevents early death of $RT6^+$ T lymphocytes based on the information from this study. But the flow cytometry data controverts the "early death" explanation. If $RT6^+$ T cells die earlier in the absence of GT and this is the only reason the $RT6^+$ T cell subset is missing, the RT6 surface marker should be expressed at normal levels on each individual cell even though those $RT6^+$ cells will be present only in small number. Prevention of early death should restore the $RT6^-$ cell population. If this were to occur, flow cytometric data would be expected to show a separate dot group corresponding to cells with normal amounts of surface RT6 protein. Our results, however, show increased RT6 staining but not a separate $RT6^-$ population. This result is more indicative of greater RT6 expression than greater $RT6^+$ cell survival. This interpretation suggests that future studies should more directly determine if GT treatment intervenes in an intracellular regulatory mechanism of RT6 protein synthesis acting on signal transduction, synthesis, modification and trafficking.

Together, analysis of cell populations (CD4, CD8 and RT6) show that GT is functional as an immunomodulator and that its effects are not global. The inhibitory effects of GT are more potent with respect to some cell types and less potent toward others. The effect of RT6 may prove to be even more selective than our results show since we did not distinguish between various subsets of $CD4^+$ or subsets of $CD8^+$ T cells. $CD4^+$ or $CD8^+$ T cells include clones with different antigen specificity and different functional characteristics. Although $CD8^+$ cells are often noted for containing CTL, a recent study indicated that one $CD8^+$ T cell subset is not cytotoxic and is protective with respect to autoimmune disease. Studies on particular $CD4^+$ and $CD8^+$ T cell subsets should be evaluated in future studies to provide a more comprehensive understanding of GT's action.

Since it is now recognized that most immunologic functions are controlled by cytokines, additional areas for further investigation involve cytokine functions which have been linked with IDDM pathogenesis and modulation by GT. Helper T lymphocytes (Th) (mostly $CD4^+$) and cytotoxic T lymphocytes (Tc) (mostly $CD8^+$) are characterized by particular patterns of cytokine production. These patterns, distinguish Th1 from Th2 among helper T cells and Tc1 from Tc2 among cytotoxic T cells. Th1 and Tc1 T cells produce IL-2 and IFN-γ which appear to be positively related to IDDM. Th2 and Tc2 T cells produce IL-4, IL-5 and IL-10 which inhibit IDDM (Mosmadd and Sad, 1996). Some evidence from the present study may indirectly link GT function to cytokine production. For example, ConA stimulation, which elicits cytokine secretion, enhanced GT-induced apoptosis. The difference in $CD8^+$ T cell percentage between GT treated lymphocytes and controls was widened by ConA stimulation. GT treatment may enhance production and function of IDDM-preventive cytokines and inhibit IDDM-promoting cytokines. This relationship may be investigated *in vitro* and *in vivo* as appropriate rat anti-cytokine probes become available.

Lymphocyte apoptosis induction by GT

There are three possible reasons to explain the observed changes among T cells. These include lymphopoiesis, selective proliferation of T-cell clones and toxic effects on specific clones. We measured peripheral leukocytes and saw no obvious alteration that was suggested an effect on lymphopoesis. Our proliferation test results showed that GT did significantly inhibit lymphocyte metabolism *in vitro* and this effect was not overcome by the mitogen, ConA. *In vivo* GT treated cells failed to show a significant response to

ConA raising the question of whether GT blocked persistently the proliferation ability of these cells. Perhaps, more notable is the finding that GT can induce apoptosis of lymphocytes both *in vitro* and *in vivo*. This provides a possible explanation for GT induced decreases in $CD4^+$ T cell percentage and emphasizes lymphocyte elimination as a likely explanation of the observed changes in T cell subsets.

Some reports in the literature indicate that GT induces apoptosis in different cell types in the immune system, including macrophages (Waring, 1990; Waring, et al., 1988), splenic lymphocytes (Braithwaite, et al., 1987) and T blast cells (Waring, et al., 1990). GT also triggers cell surface receptors and enhances lymphocyte activation (Sutton, 1995). The present investigation represents the only report which shows that GT can enhance lymphocyte apoptosis both *in vivo* and *in vitro*.

Apoptosis is an important mechanism by which the immune system eliminates harmful cells and controls its immune responses. Although several apoptosis mechanisms have been studied, Fas and Fas ligand interaction is considered among the most important for T lymphocyte apoptosis. Normally, antigen presenting cells express self antigen as a complex with MHC. This complex interacts with T cell receptors on autoreactive T cells to activate them. Activation of these T cells induces Fas and Fas ligand gene expression. T cell-T cell interaction will then cause elimination of harmful T cells through Fas ligand binding to its cognate receptor (Thomas, et al., 1995) thereby preventing development of autoimmune disease.

At present we do not know the precise mechanism whereby GT promotes apoptosis. GT may activate certain types of T lymphocytes through triggering of surface receptors. This T cell activation could increase Fas and Fas ligand expression and enhance

apoptosis. The second possibility is that GT directly binds Fas protein on the T cell surface, thereby triggering Fas receptor as Fas ligand normally would, resulting in apoptosis of target T cells. Finally, it is also possible that GT, which is relatively hydrophobic, passes through the lymphocyte cell membrane and binds specific cellular components. The disulfide group of GT may bind sulfhydyl or disulfide groups on target proteins. If these GT-binding proteins are involved in regulating the cellular processes, such GT-protein interactions could enhance apoptosis (Waring, et al, 1994). Because ConA stimulation, which enhances surface receptor production, widened the differences in apoptosis among GT treated compared to untreated cells, a direct triggering of Fas receptors by GT seems possible. This concept will require additional investigation to confirm this proposed role for GT.

Because GT induced apoptosis and altered the relative abundance of certain lymphocyte subsets, we inferred that apoptosis induction could account for T cell subset change. To verify that apoptosis is the mechanism for changing cell subpopulations we would need to demonstrate that apoptosis occurs in the same subsets of cells that we found to be decreased by GT treatment. The drawback to using the current TUNEL method is that ethanol permeabilization of the cells is incompatible with immunophenotyping because of denaturation of cellular epitopes. An alternative protocol has become available that provides the ability to directly probe apoptosis and simultaneously stain surface markers such as $CD4^+$ or $CD8^+$. This method avoids ethanol use which may diminish mAb interaction with corresponding surface markers.

Before leaving the discussion of the influences of GT on various categories of lymphocytes, we must note that the immunologic damage of IDDM takes place in the

pancreas, although several reports have indicated that central immune defects are transported to the periphery in BB rats. Attempts to document pancreatic changes, in particular changes of lymphocytes in pancreatic islets, were unproductive. In retrospect, because we conducted these studies in very early prediabetic animals, there may have been little or no lymphocytosis of the islets. A more useful method for approaching the issue of GT's effects on pancreatic lymphocytes would be to use older BB rats or to use NOD mice in which insulitis develops more chronically than it does in BB rats. The lack of definitive results from pancreatic tissue, however, does not diminish the value of observations made on splenic lymphocytes.

The work presented has shown that GT treatment is effective in IDDM prevention in DP/BB rats, presumably through selective immune function modifications. GT may have promise as protective treatment to prevent human IDDM, although GT has not yet been studied in human subjects. Perhaps *in vitro* studies of human cells would be an appropriate first step in moving toward human therapy. The ultimate goal from such investigation would be human IDDM prevention; a goal which appears reasonable in view of the positive findings in this research.

The results presented portray GT as a compound with potential for continued development as an immunopharmacologic agent for use in autoimmune diseases. We recognize that GT may function in IDDM prevention through apoptosis induction, thereby potentially affecting regulatory lymphocytes. This raises the issue of risk involved in developing GT or a similar compound for use in humans. GT did not show a serious toxicity to experimental DP/BB rats as seen in their general physical condition, histology and blood counts from treated animals. The potential value of GT as a new drug to prevent IDDM

may be rationally considered after requisite phamacology and toxicology studies are completed.

In speculating on the future potential of GT, the best example for comparison is CsA. CsA has been studied as a diabetes prevention drug. CsA is a nonspecific immunosuppresive drug which inhibits T lymphocyte activation, cytokine production, and even apoptosis. In contrast to CsA, GT, while having immunosuppressive properties, appears selective, may induce T cell activation of some cell populations and induce lymphocyte apoptosis of some cell populations. CsA interacts with cyclophilin, a 17 kDa protein that possesses peptidyl-prolyl *cis-trans* isomerase (PPIase) activity. This interaction acts by means of blockage of signal transduction. GT has a disulfide group which may bind to as yet unknown intracellular target proteins or trigger cell surface receptors to stimulate signal transduction. CsA has nephrotoxicity, manifested by decreased glomerular filtration rates, associated with a reduction in renal blood flow that reflects an increase in vascular resistance. GT showed little potential for kidney damage in our limited histologic studies. Although CsA inhibits autoimmuntity in several animal models, its toxicity currently limits its long term use. Our findings indicate that GT or a congener of GT, with appropriate pharmaceutical development, may be found to be a good alternative to CsA to intervene in cases of IDDM and other autoimmune diseases.

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LIST OF ABBREVIATIONS

APC	antigen presenting cell
Apo	cell surface protein (apoptosis)
BB rat	BioBreeding rat
BSA	bovine serum albumin
CD	cluster of differentiation
CDR	complementary-determining region
CIA	collagen induced arthritis
ConA	concanavalin A
CsA	cyclosporin
CTL	cytotoxic T lymphocyte
DP/BB rat	diabetes prone/BioBreeding rat
DR/BB rat	diabetes resistant/BioBreeding rat
ETP	epipolythiodioxopiperazine
FACS	fluorescence-activated cell sorting
Fas	name of cell surface protein family
FBS	fetal buvine serum
FITC	fluorescein isothiocyanate
FSC	forward scatter
GAD	glutamic acid decarboxylase
gld	a gene name
GT	gliotoxin

GVHD	graft vs. host disease
H&E staining	hematoxylin and Eosin staining
HLA	human leukocyte antigen
IA-2	islet antigen-2
ICA	islet cell antigen
IDDM	insulin dependent diabetes mellitus
IFN	interferon
Ig	immunoglobulin
IL	interleukin
KRB	Krebs-ringer buffer
LCM virus	lymphocytic choriomeningitis virus
lpr	gene involved in lyphoproliferation
mAb	monoclonal antibody
MEM	minimum essential medium
MHC	major histocompatibility complex
NK cell	natural killer cell
NOD mouse	non-obese diabetic mouse
PBS	phosphate buffered saline
PE	phycoerythrin
PGO	peroxidase-glucose oxidase
PI	propidium iodide
PKC	protein kinase C

PMN	polymorphonuclear leukocyte
PTP	protein tyrosine phosphatase
RIA	radioimmunoassay
RT6	a prepheral lymphocyte curface marker
SI	stimulation index
SLE	systemic lupus erythematosus
src family	name of gene family
SSC	side scatter
Tc	cytotoxic T cell
TCR	T cell receptor
TdT	terminal deoxynucleotidyltransferase
Th	helper T cells
TNF	tumor necrosis factor
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-labeling
VAF	viral antibody free

ABSTRACT

Various fungal products have immunomodulating activity and some have been studied regarding prevention of transplantation rejection. Prior to this investigation, the mycotoxin, gliotoxin (GT), has never been investigated as an immunotherapeutic drug for autoimmune disease. GT is a fungal secondary metabolite and a member of the epipolythiodioxopiperazine (ETP) family which has been shown to inhibit phagocytosis, induction of cytolytic T cells and the proliferation of T cells following mitogen stimulation. GT also induces *in vitro* apoptosis in certain immune cell types. More importantly, GT exhibits selective activity towards cells of hemopoietic origin.

Autoimmune diseases are disorders caused by immune responses to self antigens. Insulin dependent diabetes mellitus (IDDM) is an organ-specific autoimmune disease in which insulin secreting pancreatic islet β-cells are destroyed leading to hyperglycemia, ketoacidosis and various systemic complications. Because of its potential effects on the immune system, we evaluated GT for its ability to prevent IDDM. This study is the first to successfully use GT to prevent an autoimmune process.

GT prevented IDDM in spontaneously diabetic DP/BB rats without causing significant adverse effects among the treated animals. GT treated rats developed diabetes at a rate of 55% by 120 days of age compared to 90% for control rats. GT treatment also significantly decreased serum glucose levels from an average 278 mg/dl to 185.67 mg/dl among non-diabetic/pre-diabetic animals.

A series of studies was conducted on 65 days old DP/BB rats, prior to development of diabetes to phenotypically characterize the splenic lymphocytes recovered from

animals chronically treated with GT. A parallel study examined the direct effects of GT on splenocyte preparations incubated with this mycotoxin.

This study found that GT selectively affects certain lymphocyte subsets. Animals treated with GT showed involution of splenic follicles and several effects on lymphocyte subpopulations were found. *In vitro* treatment of splenocytes with GT revealed decreased $CD4^+$ and increased $CD8^+$ T cell subsets. $CD8^+$ T cells function as an important regulator of autoimmunity, especially influencing the activity of $CD4^+$ T cells. GT effects on $CD4^+$ and $CD8^+$ T cells are consistent with changes anticipated to inhibit IDDM pathogenesis. *In vivo* treatment with GT did not result in detectable alterations in relative $CD4^+$ and $CD8^+$ cell subsets, although this may have been more related to pharmacologic reasons than the physiological effects of GT.

Importantly, this study found that both *in vitro* and *in vivo* GT treatments significantly enhanced the detectable RT6 surface marker. The $RT6^+$ T cell subset is a key regulatory element in IDDM pathogenesis. Increased numbers of RT6 surface markers may be involved with IDDM prevention or may be a result of it.

GT induced lymphocyte apoptosis among spleen cells from DP/BB rats was altered *in vitro*. The average increase in apoptotic cells due to GT treatment was nearly four fold. Results from this study suggested that the mechanism whereby GT prevents IDDM in DP/BB rats is through apoptosis. Coupled with the finding of altered lymphocyte populations, it may be suggested that apoptosis of regulatory cells, or effector cells is involved in diabetes prevention in this system. The finding that $CD8^+$ cells and NK cells which include cytotoxic effectors that can promote pancreatic damage, were not decreased

by GT treatment suggests that the effects may reside with regulatory cells rather than with effectors, although additional study is warranted to fully understand this process.

This research is the first to show that GT has a protective effect against an autoimmune disease. We also found that GT is a selective immunomodulator altering the ratio of $CD4^+$ and $CD8^+$ lymphocytes and causing increased $RT6^+$ surface marker to appear as an important subset of lymphocytes. This study is also the first to demonstrate that apoptosis due to GT treatment occurs in intact animals.

Because indicators of systemic toxicity showed that GT is relatively benign in experimental animals, as evidenced by lack of irreversible histopathology, normal weight gain and normal leukocyte counts, and it has a beneficial effect on IDDM development, GT should be considered for continued evaluation as a potential IDDM preventive drug.