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**THE ROLE OF β -CELL APOPTOSIS IN DIABETES PATHOGENESIS IN
BIOBREEDING/WORCESTER DIABETES PRONE RATS**

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ABSTRACT

The purpose of this project was to correlate the time of onset of pancreatic β -cell apoptosis with the onset of diabetes in the BioBreeding Diabetes Prone (BB/DP) rat model to provide an initial determination of whether apoptosis is a primary or secondary event in the disease onset. Immunofluorescence microscopy was used to assay the appearance of Annexin-V apoptotic protein marker and the appearance of DNA fragmentation (via TUNEL assays). Due to time constraints, confirmation of β -cell apoptosis was not possible in this study; future research, however, should conclusively prove the role of β -cell apoptosis in diabetes onset.

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BACKGROUND

Insulin-Dependent Diabetes Mellitus (IDDM)

Metabolism is an exceedingly complex process among higher organisms. As such, there are many steps which can be tampered with, retarding or even halting the process. Diabetes is a disease resulting from a set of these failures, particularly those relating to the role of insulin in cellular glucose uptake. Insulin, a hormone secreted by cells in the pancreas, can be prevented from functioning in many ways, and the various types of diabetes correspond to each of these errors. The focus of this project is principally insulin-dependent diabetes mellitus (IDDM), otherwise known as Type 1 diabetes.

Disease Description

As stated above, diabetes can result from errors at a number of steps in glucose metabolism. The most common cause of the disease is an inability to produce or use insulin. Insulin is a polypeptide hormone secreted by cells in the Islets of Langerhans, located in the pancreas. First described by Paul Langerhans in 1869, these islets contain the cells which produce insulin, termed β -cells (Matthews, 2003). Insulin-dependent diabetes results when these β -cells are destroyed, and thus unable to produce insulin. The consequence of this damage is most directly observed in the elevated blood glucose levels of affected individuals. Although blood glucose concentration is high, cells are unable to absorb and metabolize glucose without the presence of insulin. This results in a group of symptoms that characterize the disease (NIH, 1994). One of the first typical symptoms of IDDM is increased urine output. High blood glucose concentration causes

some glucose to spill into the urine. Glycosuria (high urine glucose) is, in fact, one of the primary indicators that a patient may be diabetic. In response to this high concentration of glucose, more urine is produced in order to flush the glucose out of the patient's system. This increase results in dehydration and increased thirst, despite drinking water in excess (Chase, 1992).

Other symptoms of the disease include significant weight loss and fatigue, regardless of the amount of food eaten by the patient. These symptoms are caused by the inability to use blood glucose. In response to a perceived shortage of energy supply, the body turns to fat and protein as alternative sources of energy which results in a decrease in the amounts of both fat and muscle, and accounts for the weight loss characteristic of IDDM patients. The lack of ability to use glucose as an energy source results in lessened energy and fatigue (NIH, 1994).

If allowed to progress unchecked, IDDM is fatal to affected individuals. The breakdown of fat and protein discussed above results in byproducts called ketones that build up in the blood, causing acidosis (high blood acid level) to create a condition called diabetic ketoacidosis (DKA) which can lead to coma and subsequent death if left untreated (Matthews, 2003).

Prevalence

As diabetes is a serious and life-threatening disease, it is important to consider how many people are affected by the illness. The most direct method to measure this impact is to study the number of people affected. As of 2002, 18.2 million people – 6.3% of the population of the United States – have diabetes (American Diabetes Association, 2002). Five to ten percent of diabetics – up to 1.8 million – have Type 1 diabetes, onset

of which is characteristic of children and adolescents. The remainder have type 2 diabetes, characterized by a diminished sensitivity to insulin. Though increasingly prevalent in the United States, the occurrence of diabetes is increasing worldwide.

An additional method of quantifying the effects of diabetes is to examine the expenses incurred by the disease. *Diabetes Care* commissioned the American Diabetes Association (2002) to conduct a study of the economic costs of Type 1 and Type 2 diabetes. In 2002 alone, direct and indirect expenses totaled \$132 billion spent on diabetes treatments and complications. Direct expenditures include health care, and treatment of diabetes and its complications. A total of \$92 billion (70% of the total cost) was directly attributable to diabetes (i.e. did not include costs to family caregivers, loss of income, etc.). In addition to effecting significant health care expenses, diabetes causes a marked increase in mortality. This increased mortality brings about a loss of income, as affected individuals are unable to work. This phenomenon is accounted for under the indirect expenditures, comprising \$40 billion.

Worth noting is the fact that although diabetics comprise only 6.3% of the U.S. population, over ten percent of the \$832 billion spent on healthcare can be attributed to treatment of the disease. The 2003 study also found that healthcare costs were, on average, 2.4 times the healthcare expenses for non-diabetics. This disparity is an unambiguous indicator of the impact of diabetes in the United States.

Current Treatments

As Type 1 diabetes affects a great number of people, much attention is devoted to treatment of the disease. However, treatment of diabetes presents several challenges. Effective treatment of the disease mandates strict monitoring and regulation of blood

glucose. Many patients become frustrated with the constant pressure and difficulty of chronic disease care. In addition, subcutaneous injection of insulin remains the most proven and reliable treatment for IDDM. Patients who have difficulty administering insulin suffer a decrease in the quality of their care (Matthews, 2003). In light of the difficulties encountered by the current solution, improving treatment for IDDM is one of the most investigated areas of diabetes research.

Because insulin therapy remains the most proven and the least invasive of treatments, significant effort has been devoted to optimization of injection of the hormone. The research conducted to this end has had several benefits, not the least of which is the development of different kinds of insulin. The insulin first used for therapy was isolated and purified from cow and pig pancreata (Morrison, 2003). Development of recombinant DNA technology allowed the development of strains of *E. coli* that produce the human insulin used by most Type 1 diabetics. In addition, the purified hormone can be crystallized to create a suspension – the result is a longer-acting insulin, commonly used as a basal dose of the hormone (Eli Lilly, 2004).

Although the creation of new kinds of insulin resulted in increased adaptivity of treatment to patient lifestyle, the ideal solution is a closed system capable of replicating the regulation of blood glucose and insulin production conducted by the pancreata of non-diabetics. One step toward such a system was the design of the insulin pump. Now becoming a popular alternative to traditional injection therapy, insulin pumps provide a constant basal rate of short-acting insulin, given subcutaneously through a catheter. When the patient eats, boluses of insulin may be given to compensate for the resulting increase in blood sugar, a process which can be likened to the signaling of healthy pancreatic β -

cells to produce insulin (Matthews, 2003). To be sure, design of this system was a major advance in replicating the behavior of a healthy pancreas.

Pump therapy, however, is not yet the ideal solution; the patient must refill the insulin reservoir and remains responsible for monitoring blood glucose levels. Research on devices to measure blood sugar and adjust insulin injection accordingly within one system is currently underway, but other alternatives are currently being tested. Islet transplantation has been conducted with mixed success, but the risk remains high for most patients. Patients with “brittle” diabetes can succumb to insulin reactions, or diabetic seizures, even with exacting, strictly regimented control (Morrison, 2003). Islet transplantation has been administered on several patients at the Heritage Medical Research Centre in Edmonton, Alberta. 12 patients were examined and monitored for a 2001 study released by the University of Alberta. Of these 12 patients, 11 were able to attain insulin independence after the procedure, and dependence on insulin was significantly reduced for the remaining patient (<10U/day). In addition to insulin independence, blood glucose concentrations were significantly reduced. Three patients had minor complications during surgery (thrombosis of a peripheral branch of the right portal vein, bleeding from the hepatic needle puncture site) but these problems were fully resolved. Two patients showed transient vitreous hemorrhages. Keeping these complications in mind, for patients that have diabetes that is difficult to control, the risk-to-benefit ratio is in favor of islet transplantation (Ryan et al, 2001). For the majority of patients, however, the risk of surgery far exceeds the risks of complications associated with traditional insulin therapy.

As a result, diabetes is among the foremost researched diseases in the world. This is principally for two reasons: the condition affects so many people, and it is a chronic illness with an overwhelming amount of responsibility for care placed on the patient. The research so far has benefited afflicted individuals in many ways - treatment can now adapt to individual lifestyles, as opposed to the strict regiments prescribed during earlier treatments; new types of insulin allow better mimicry of a healthy beta-cell response; and transplantation has served as a solution for patients whose conditions prove difficult to control. Each of these advances in the field has helped diabetics to lead healthier, longer lives. However, in spite of these advances, the causes of and cure for the disease remain elusive.

IDDM as an Autoimmune Disease

As many questions relating to insulin-dependent diabetes mellitus remain unanswered, the disease is a subject of prolific research. Subjects in question range from environmental stressors that may have a role in onset, to possible therapies for a cure. Although the exact biochemical pathways leading to IDDM onset are not yet known, there is a consensus as to the general cause of the disease. It appears as though the immune system is triggered to destroy pancreatic β -cells, causing a lack of insulin and resulting hyperglycemia. Finding the trigger of this process is the very foundation for a great deal of current diabetes research.

Autoimmunity

The immune systems of higher mammals are complex machines that serve many purposes. For immunity to work correctly and efficiently, many conditions must be

satisfied, and this is often the case. However, there exist sets of circumstances that, when acting in concert, can have profound effects on the immunity of an organism, perhaps even threatening its life (Nairn and Helbert, 2002). Such is the case with diabetes – there are many sets of conditions that can result in onset of IDDM, all of which seem to have one common result. This commonality is the destruction of insulin-producing β -cells by auto-reactive T-cells, and in fact defines the very premise of IDDM.

There are several pathways in which autoimmunity can be triggered or induced within an organism. In summary, an over-sensitized T-cell mistakes a protein or polypeptide fragment of host origin for one of foreign origin. The cell begins a course to rid the host of this mistaken protein, and triggers an immune response. The cells activated in this response are specifically targeted to the protein, and a highly effective and specific attack of cells expressing it is subsequently carried out (Nairn and Helbert, 2002).

As might be expected, the result of such a precise attack is practically complete elimination of the targeted cells. When the protein is a genuinely foreign antigen (Ag), this process works to the organism's benefit, fighting off infection from a myriad of harmful microorganisms. However, when the targeted set of cells comprises a vital part of the organism, the immune system effectively cripples its host. The steps presented below in Figure 1 present an approximate course of events in diabetes pathogenesis, and are illustrated in the context of diabetes. Specific proteins and antigens, however, remain elusive in developing a robust mechanism for the origin and development of the disease in this particular diagram.

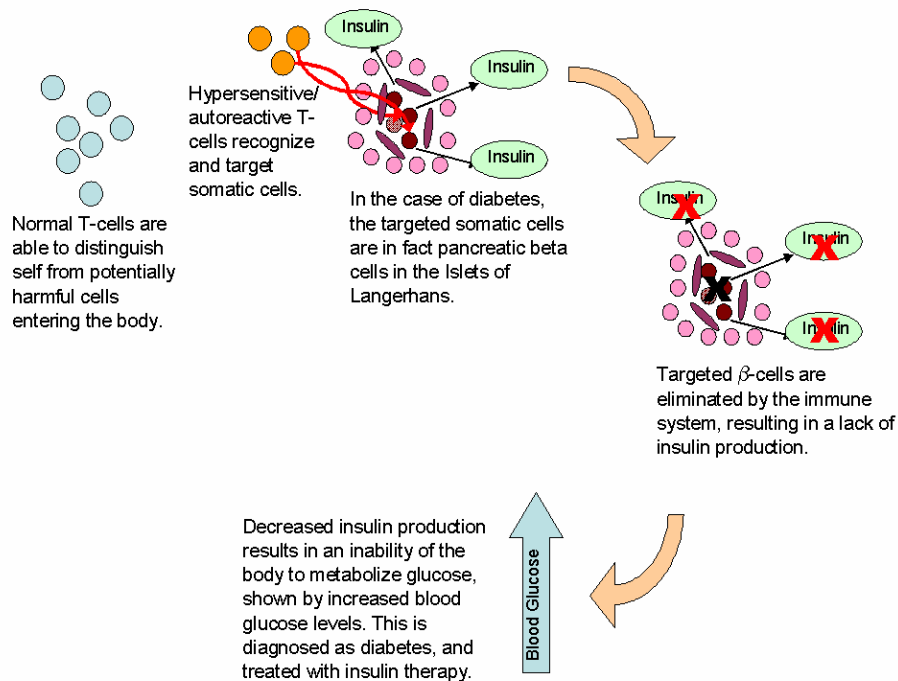


Figure 1: Schematic Representation of the Autoimmune Response as it Relates to Diabetes.

Autoreactive T-cells enact an ultimately cytotoxic response against β -cells of the host organism, effectively eliminating insulin production, and thereby causing diabetes.

GAD as a Primary Autoantigen in IDDM, and its Gene Therapy

Relatively few proteins have been subject to thorough investigation with regard to their roles in Insulin-Dependent Diabetes Mellitus, but studies of glutamate decarboxylase (GAD) have proven particularly interesting. It has been shown in humans, in the non-obese diabetic (NOD) mouse, and in the BioBreeding (BB) rat that expression of GAD as an autoantigen is demonstrably increased among newly diabetic and high-risk individuals (Hawa and Leslie, 2002). Appearing as early as eight years before onset of the disease, antibodies against GAD are an effective marker for type 1 diabetes (Yoon et al, 2000). Although it is evident that GAD plays some role in diabetes pathogenesis, whether that role is primary or secondary has been under speculation for some time.

The NOD mouse is the model in which most of GAD research has been conducted, and has also shown to be the most accurate animal of type 1 diabetes to date. GAD acts as a β -cell antigen, and researchers have questioned whether this phenomenon occurs before or after the immune response. Evidence in the NOD mouse suggests that the former is true, as treating mice of varying age with plasmid encoding GAD successfully prevented diabetes onset (Fig. 2) (Balasa et al, 2001). Though it appears promising, attempts to duplicate the feat in rats were unsuccessful (Petersen et al, 1997). As a result, no conclusive link has been made between glutamate decarboxylase as an autoantigen and the development of diabetes.

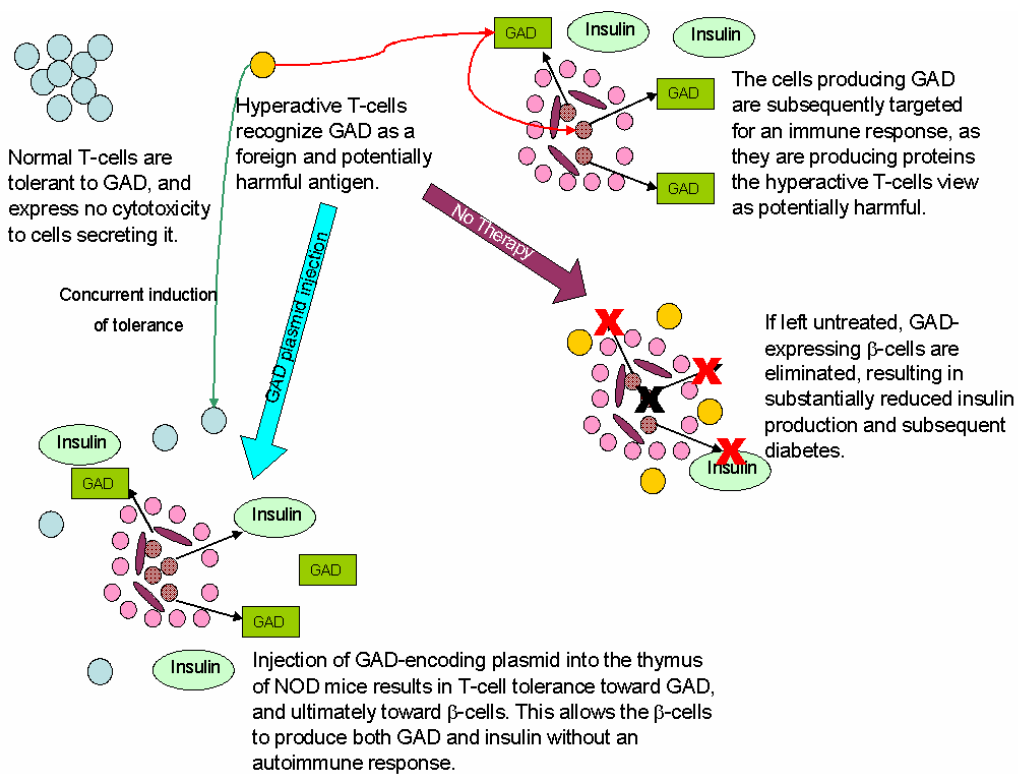


Figure 2: GAD Gene Therapy. Tolerance of the proposed autoantigen GAD may be induced in NOD mouse T-cell populations via injection of GAD-encoding plasmid into the thymus. These newly tolerized T-cells will no longer target GAD and the β -cells that produce it for destruction by the immune system.

Autoimmune Trigger Hypotheses

The link of the diabetes autoimmune response to an antigen, in fact, has not been made for any single protein. After decades of research, the seemingly elementary question of the cause of diabetes remains unanswered. There are several hypotheses, however, which provide reasoning for documented scientific data (Fig. 3). The general theme of these hypotheses is consistent: they are each based on the idea of IDDM as an autoimmune disease. The immune system becomes hostile toward β -cells in the pancreas – cells that would normally be considered as self and therefore harmless – resulting in their subsequent destruction. This process results in the development of diabetes. The variable among these hypotheses is the trigger – what causes the immune system to become autoreactive? Finding the answer to this question has been, and remains, the objective of countless studies and investigations.

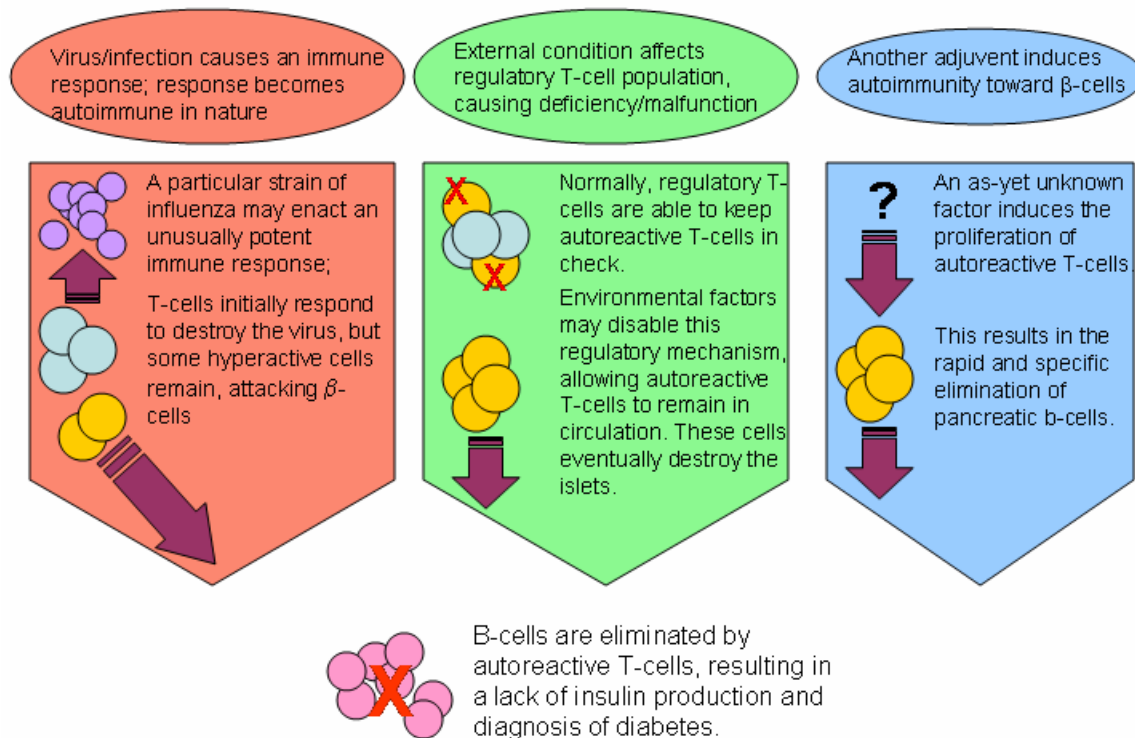


Figure 3: Potential Causes of Autoimmunity. A number of possible causes exist for autoimmune reactions; many diabetes researchers ask the question, which trigger is responsible for IDDM?

Although this question drives so many research goals, determining the causative factor is not a simple task; there are a number of conditions and mutations that are capable of predisposing an individual for diabetes. In short, the pathogenesis does not occur in one set path, but in myriad ways, each mechanism bearing a subtle resemblance to the last. As such, the most reasonable method of finding the cause or causes of the disease may rest in researching more about the convoluted mechanisms at its core.

Role of Apoptosis in IDDM

Apoptosis, or programmed cell death, is a process that has been enlightening in diabetes research to date. Problems related to apoptosis are broad in range, as a disturbed

pathway may disable or accelerate the process, causing some forms of cancer or premature death, respectively. The depth of its role in type 1 diabetes is under current investigation, and this MQP will serve as further investigation to that end.

Apoptosis

As defined above, apoptosis is controlled cell death. This is a crucial mechanism at any stage in the life of a healthy organism. At the embryonic stage, the process is used as a tool to govern the carefully timed development of the various systems of the body. The “sloughing off” of cells from the embryo which occurs at several stages of development is characteristic of a wave of apoptosis, necessarily enabling energy and resources to be directed toward the remaining, living cells of the embryo. After birth, apoptosis is similarly used throughout development of the organism to adulthood, as a mechanism of shaping, directing, and developing its systems, from circulatory to reproductive. After reaching adulthood, its prevalence may seem less pronounced, but apoptosis is still used to keep all the various systems in check and to prevent disease (Yin and Dong, 2003).

The general mechanism of apoptosis is governed by a signaling pathway. One of a number of external conditions or signals causes activation of the apoptotic pathway (Fig. 4). This event may be the presence or absence of a certain nutrient, or the binding of a specific protein to a receptor on the target cell membrane.

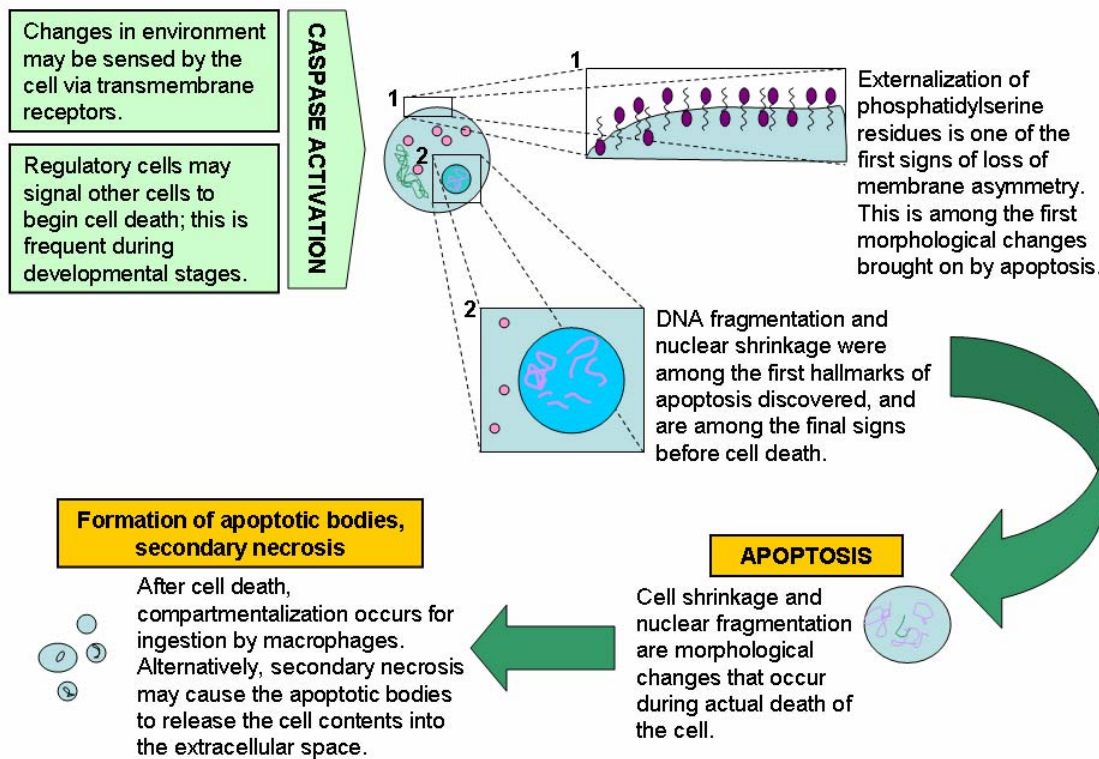


Figure 4: Apoptosis Overview. A number of events external to the cell can initiate a signaling pathway that results in caspase activation within the cell. After this occurs, caspases work to destroy the cell while minimizing damage to neighboring cells. Minimal damage to adjacent cells is achieved through compartmentalization of the dead cell and targeting for phagocytosis. Alternatively, secondary necrosis may occur, spilling cellular contents into extracellular space.

The second general step of the process is the upregulation and/or activation of one or more of a set of proteins known as caspases. Caspase activation typically consists of pro-caspase cleavage, and heralds the destruction of the cells in which it occurs (Smolewski et al, 2003). Morphologically, apoptosis is generally characterized by compartmentalization of the cell into smaller fragments. These fragments, termed apoptotic bodies, aid in phagocytosis of the cells by macrophages. If there are too many apoptotic bodies for the macrophages present, compartmentalization is followed by secondary necrosis, releasing cellular contents into extracellular space.

Apoptotic Biochemical Markers

When a cell undergoes apoptosis, several telltale events occur which can be used to detect the process and to learn more about its cause. One of the signs of programmed cell death is DNA fragmentation (Gilchrest and Bohr, 2001). This can be observed by staining the cells and checking for the presence of nicked DNA. This experiment is typically performed via a TUNEL assay. In this assay, terminal deoxynucleotidyl transferase (TdT) catalyzes the incorporation of bromodeoxyuridine (BrdU) onto fragmenting residues of DNA by nick-end labeling. The BrdU may initially be tagged with a fluorescent marker, or can be subsequently stained to preserve results. TUNEL is a highly specific assay, but can not be used in a live assay, as cells must be lysed (and therefore dead) to conduct the experiment.

Another sign of apoptosis is cell shrinking, which may be observed by light microscopy (Yin and Dong, 2003). Unfortunately, this is one of the later signs, and so its use is limited in finding the cause of apoptosis. Mitochondrial membrane potential is also affected by the process, and its alteration can serve as an early marker of cell death. This can be detected in living cells with the use of cationic fluorochromes. Their distribution can be monitored via flow cytometry or fluorescence microscopy, and thus can be used to find information about membrane potential throughout the process of apoptosis. One caveat of this indicator is that it is not always affected by apoptosis, and there are several pathways that circumvent this event as a secondary effect.

Caspase activation is another event that may be detected and found as evidence that apoptosis is occurring in sample cells. Caspases are enzymes involved in the destruction of cells. There are two general classes – initiator caspases and effector

caspases. The initiators are responsible for the initial response to an apoptotic signal. These enzymes, in turn, activate the effectors, which are responsible for breaking down the cell. Caspases can be tracked via fluorescent markers, and monitored by flow cytometry (Stein et al, 2004). There are, however, multiple initiators and effectors, and the result is a great number of possible pathways concluding in apoptosis. As such, learning about the pathway using this method may be time-consuming, but is extremely useful in divining the origin of cell death in cases where some general information regarding the cause of death is known before investigation begins.

One event characteristic of all apoptotic cells, however, is a loss of membrane asymmetry shortly after initiation of cell death. Of particular value is the flipping of phosphatidylserine residues from the cytosolic portion of the membrane to the extracellular portion (Williamson and Schlegel, 2002). With the use of Annexin-V, a phosphatidylserine-binding protein, and a fluorescent marker, it is possible to conclude whether cells are undergoing apoptosis on a very general level by measuring an increase in Annexin-V binding. This assay is very useful in determining whether programmed cell death is occurring, and if so, which cells are affected.

Apoptosis and Autoimmune Disorders

As cell death can result in extrusion of cellular components, it is capable of having a profound effect on the immune system. Adaptive immunity is a necessarily complex system, and the breadth of foreign matter it must protect against is nothing short of remarkable. Taking this into consideration, a simple error in distinguishing “self” from “non-self” when dealing with apoptotic bodies is quite capable of setting the immune system against its host. Alternatively, harmful effects can also be caused by a lack of

apoptosis. If the growth of certain infected or cancerous tissues is allowed to continue unchecked, it can result in serious complications. The consequences of both overactive and diminished apoptotic mechanisms can result in many types of illness, ranging from cancers to autoimmune diseases.

Strong associations have already been made between programmed cell death and diseases known to be autoimmune in nature. Systemic lupus erythematosus (SLE) is one such disease. SLE is characterized by pronounced alterations to the immune system, eventually effecting autoimmunity against a number of organs. In the case of lupus, apoptosis has been found to generate nucleosomes that act as major antigens in the disease. In addition to this development, nucleosomal antibodies are highly specific for SLE. This means that apoptosis is capable of accelerating the disease, if it is not in fact the primary cause. Additionally, there is a sort of positive feedback mechanism in terms of the antigens (Ags) produced. A greater number of cells undergoing apoptosis translates to more autoAgs produced. These nucleosomes also inhibit the phagocytosis of other apoptosing cells. This results in additional release of autoAgs, as the apoptotic load becomes too great for phagocytes to properly digest (Kaplan, 2004). This self-perpetuating cycle is central to the problem of SLE, and so learning more about the causes of apoptosis as well as preventative mechanisms will provide a great deal of enlightenment on the disease.

As amplified apoptosis seems a basic problem in SLE, so might restricted apoptosis induce illness in the case of rheumatoid arthritis (RA). Numerous signaling proteins have been detected in joints affected by active RA. A number of these proteins are key elements in *anti*-apoptotic mechanisms. This has led researchers to believe that

the introduction of pro-apoptotic elements may be therapeutic in treatment of the disease. Experimental data shows that increasing the amount of apoptosis in the affected area may reduce pain and inflammation (Pope and Liu, 2003). Again, coming to a greater understanding of the mechanism of the disease and the role of apoptosis in that mechanism may aid the development of therapy for the disease.

Apoptosis and IDDM

Programmed cell death and its consequences seem to be at the heart of many disorders involving autoimmunity, and IDDM is no exception (Fig. 5). Determining that role in the case of diabetes, however, has proven more trying than one might expect. There are a variety of theories surrounding this issue in current research, but the basic question is whether β -cell apoptosis is a primary cause of the disease. Were this true, the solution for the disease would likely lie in the mechanisms determining self from non-self. Whether searching for an error in the human major histocompatibility complex (MHC) or a complication in antigen presentation and dendritic cell proliferation, finding the defect primarily responsible for onset of the disease will allow developing a treatment to become that much more attainable.

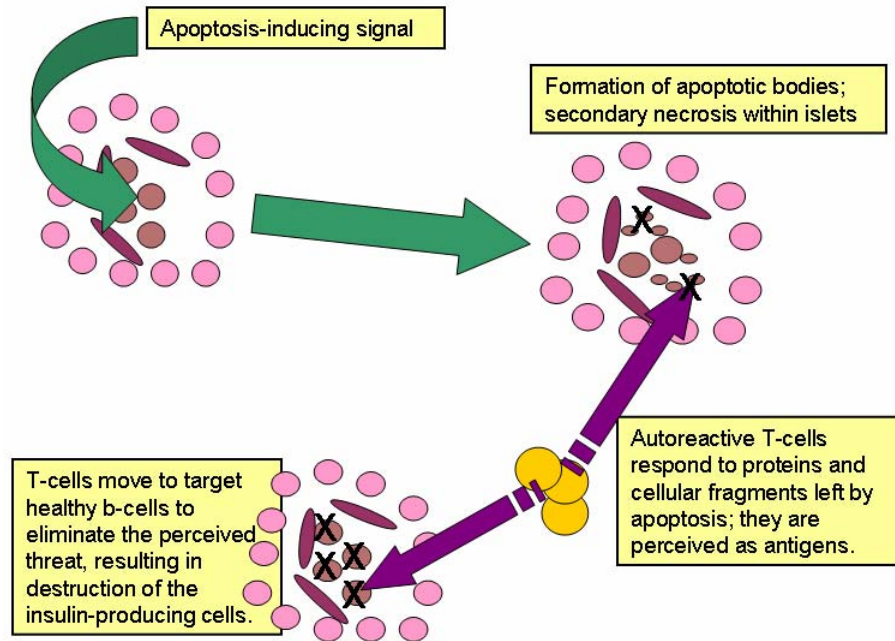


Figure 5: Apoptosis as a Primary Cause of Diabetes. It is hypothesized that β -cell apoptosis may be primarily responsible for the autoimmune response characteristic of diabetes. Apoptotic bodies or proteins left by secondary necrosis initiate an immune response that results in islet cell destruction, effectively eliminating production of insulin.

The Major Histocompatibility Complex (MHC) and Diabetes

The MHC of an organism refers to the genetic region encoding a group of proteins qualified as antigen receptors. These proteins, along with T-cell receptors and antibodies, are primarily responsible for distinguishing between self and non-self. In humans, the MHC genes are known as Human Leukocyte Antigen (HLA) genes. Their protein products are referred to as HLA molecules (Nairn and Helbert, 2002). In short, if the HLA genes of diabetics encode a molecule that binds to some product of β -cell apoptosis, a defective human MHC may be the origin of IDDM.

Role of Antigen-Presenting Cells (APCs) in Diabetes

The scenario presented above is one of several possible pathways for diabetes induction, but another autoimmune issue may also lie in the mechanism of antigen presentation among diabetics. APCs are responsible for assimilating antigen particles into their membranes and presenting them to T-Cell receptors (TCRs). Upon recognition by a TCR, the body is able to launch an adaptive immune response against the foreign antigen. The problem that may be at work in the case of IDDM has to do with which antigens these cells eventually present to the rest of the immune system. The cellular fragments produced by a cell undergoing apoptosis are usually subject to phagocytosis (Nairn and Helbert, 2002). If these macrophages ingest β -cell apoptotic bodies and subsequently present some part of the β -cell as “non-self,” they may be responsible for initiating the immune response against islet cells. This immune response would, in turn, initiate IDDM in an otherwise healthy individual.

Rodent Models for Diabetes

Ideally, any research in an effort to learn more about a disease affecting humans would take place with humans. In actuality, however, there are a myriad of reasons this is not possible. The ethics of such experimentation are highly suspect for one, but it would provide several challenges even without that as an issue. The relatively long gestational and maturation periods for humans make the species a poor candidate for repeated experiments, multiple generation studies, and determining new directions in research. Fortunately, humans are not the only mammals afflicted with diabetes; in fact, the illness has been reported in many species, from mice and rats to cats and dogs.

While many types of animals may exhibit the disease, the ideal species for the research required should have a relatively short lifespan, but should operate on a timetable capable of human observation. Higher mammals are less useful in this respect, for reasons similar to humans. Rodents, however, meet this requirement with the additional advantage of reduced size and living requirements. In modern diabetes research, mice and rats are the primary models under investigation. These models both provide a high number of offspring, typical lifespans of well under 5 years, and have demonstrated high levels of consistency when breeding for a certain trait. Two of the most popular models to date are the non-obese diabetic (NOD) mouse, and the BioBreeding Diabetes Prone (BB/DP) rat (which includes the BB Wistar rat and the BB/Wor rat). Both of these models provide areas of great homology with humans, and complement each other quite well. Because the BB/DP rat seems to closer imitate human diabetic responses and treatment, it was the model chosen for this study.

Discovery

Spontaneous diabetes in the BB Wistar rat was first noted in 1974 at BioBreeding Laboratories in Ottawa, Ontario, Canada in a non-inbred colony of Wistar rats. These rats were maintained with daily injections of insulin, and a breeding colony was successfully established in 1977. In addition to the Ottawa colony, a successful inbreeding program was carried out at the University of Massachusetts Medical School funded by the National Institutes of Health. The eventual successors of the inbred Worcester colony (known as the BB/Wor rats) are the subjects used in this study (McNeill, 1999).

Of the BB/Wor (Worcester) rats, there are two types; the diabetes-prone (DP) animals, of which more than 90% spontaneously develop diabetes, and the diabetes-

resistant (DR) animals, which do not develop diabetes. These rats are often used as a control strain in demonstrating differences between rats affected by diabetes and their unaffected counterparts.

Past Uses of the BB/Wor Rat

The BB/Wor rats have been used by a number of researchers in elucidating the mechanism behind diabetes pathogenesis. To this end, studies have enabled the determination of a number of immunological factors relating to the disease and its progression. For example, the role of natural killer (NK) cells has been investigated conclusively in the BB/DP rats. NK cells are more prevalent in the DP strain than in the DR strain, and an increased incidence of NK activity is consistently found in the inflamed islets during the initial stages of the disease (Woda et al, 1986; Hosszufalusi et al, 1993). This corresponds with the human case, as increased levels of NK cells and corresponding activity have been found in human IDDM as well. Additionally, NK cells showed spontaneous cytotoxicity to pancreatic islet cells *in vitro*. This data would seem to implicate the NK cells as responsible for β -cell destruction, but further research proved otherwise. Specifically, it was found that *in vivo* depletion of the natural killer cells did not prevent diabetes; autoimmune targeting and destruction of pancreatic β -cells can occur in the absence of NK cells (Edouard et al, 1993). Subsequent adoptive transfer experiments showed T-cells within a lymphocyte population mediate the autoreactivity.

Another area of substantial research as to the cause and progression of diabetes includes the mechanisms of the cytokines used in regulating the autoimmune attack of β -cells. Cytokines are molecules that enable the cell-to-cell communication necessary to mediate an immune response. It has been suggested that β -cell damage due to infiltrating

leukocytes is caused by cytokines. The balance of these cytokines seems to be crucial to the autoimmune response. In particular, interferon-g, IL-12, and IL-2 have been identified in pancreatic islets of BB/DP rats both before and after onset of diabetes. The cytokine balance of islets in both rats and humans has led researchers to believe that a strong inflammatory response is in abundance over a suppressive immune response (Katz et al, 1995). Regarding the nature of cytokines toward islet cells in particular, IL-1 and TNF-a have been found to be cytotoxic to human islet cells *in vitro* (Rabinovitch et al, 1996). BB lymphocytes exhibit increased IFN-g and IL-2 release when cultured with allogenic islet cells. Th2 (suppressive) cells secrete the cytokines known as IL-4, IL-5, and IL-10, each of which counter the inflammatory, cytotoxic immune response. In light of this BB data, it is evident that a proper balance of regulatory (T-reg) and cytotoxic T-cells is necessary to prevent the disease.

The roles of NK cells, T-cells, and cytokines in diabetes pathogenesis are merely three relatively specific areas of research regarding diabetes in the BB rat; there are many articles in the literature ranging from the proteins expressed in the membranes of T-cells in affected rats, to the energy metabolism of immune cells in general in BB/DP rats in comparison with their DR counterparts. The BB rat has proven exceedingly useful in determining the nature of diabetes and the factors behind its incidence in humans. As such, it serves as a good model of the disease, and would do well in further researching the cause of diabetes.

Role of the BB/Wor Rat in the Current Project

The BioBreeding rat will be used in this project to study the role of apoptosis in developing diabetes. The mode of this use will be to examine pancreata of a number of rats at various time intervals. If apoptosis indeed occurs within the islet cells, this time-course will allow its timing to be recorded in conjunction with occurrence of diabetes.

PROJECT PURPOSE

The purpose of this project was to use immunofluorescence microscopy to correlate the time of onset of β -cell apoptosis with the onset of diabetes in the BB/Wor rat model. Tracking the level of apoptosis in a number of rats over time will provide an elementary determination of the role that programmed cell death plays in the development of diabetes. As such, this research will help to discern whether the role of β -cell apoptosis is primary or secondary in diabetes pathogenesis.

METHODS

Rat Tissue Preparation

BioBreeding/Worcester (BB/Wor) rats of both diabetes prone (DP) and diabetes resistant (DR) strains were raised at the UMASS Medical School Animal Facility. Cervical lymph nodes (CLN) and pancreata were extracted from the rats. Single cell suspensions were prepared of the rats' cervical lymph nodes for the Annexin-V assay. CLN and pancreatic sections were prepared and put on slides for the TUNEL assay. Pancreata and lymph nodes were placed in cassettes and preserved with Bouin's solution and 10% paraformaldehyde. Pancreatic and CLN sections were then made by the UMMS cell morphology core.

Annexin-V Staining

Annexin-V staining is an apoptosis detection assay that differentiates cells undergoing programmed cell death from normal, healthy cells by showing a lack of membrane asymmetry. Phosphatidylserine (PS) residues are normally restricted to the cytosolic portion of the cell membrane. During the initial stages of programmed cell death, however, these residues can be found on either side of the cell membrane, thus Annexin-V is then able to bind the PS residues on apoptotic cells that have rearranged their cell membranes (Figure 6). Annexin-V positive cells can then be detected via fluorescence-activated cell sorting (FACS), as the Annexin-V is conjugated to the green-fluorescing FITC for detection.

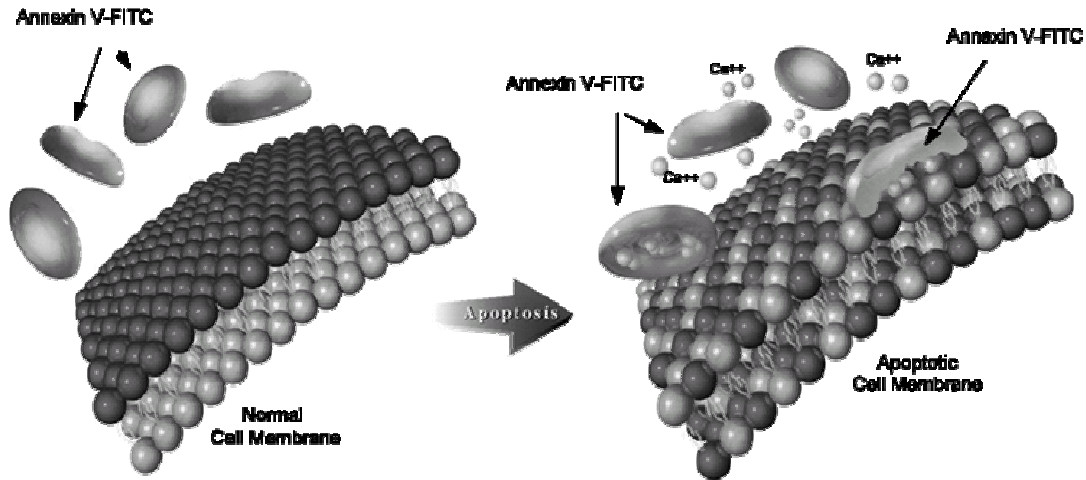


Figure 6: Visual overview of the Annexin-V Assay. Annexin-V binds PS residues, which are externalized only in apoptotic and necrotic cells. Visualization of Annexin-V binding is possible through the use of fluorescence-activated cell sorting (Southern Biotechnology Associates, 2004).

Although Annexin-V staining can conclusively show membrane asymmetry, it is not capable of differentiating between viable and necrotic cell populations. Because of this, Annexin-V assays are often coupled with a cell-viability assay to be evaluated in tandem with Annexin-V data. Propidium Iodide (PI) is useful in this respect, as it binds nucleic acids. When PI is conjugated to a fluorescent protein, it is possible to check the necrotic cells against those that are viable in FACS analysis. Combining PI-binding with the Annexin-V assay, it is possible to distinguish between healthy, apoptotic, and necrotic cells.

Lymph Node Preparation for FACS Analysis

Cervical, mesenteric, and pancreatic lymph nodes were extracted from BB/DP and BB/DR rats for comparative analysis. Fat external to the lymph node was removed, followed by temporary storage in RPMI on ice. Using a CELLECTOR™ tissue sieve, a

single cell suspension was made in RPMI for each type of lymph node, and put through a 70- μ filter. This was followed by centrifugation at 300g for 6m at 4°C to pellet the cells.

Cell counts were made for each sample, and also served as preliminary viability assays, as they were made with a hemacytometer in the presence of Trypan Blue. Trypan Blue is excluded from viable cells, and stains necrotic ones. Approximately 10^6 cells were aliquotted per vial, adding 2mL FACS buffer to each vial. FACS buffer is composed as follows: 0.1% BSA/ 0.1% NaN_3 in Dulbecco's Phosphate-Buffered saline without CaCl_2 and MgCl_2 . Samples were then centrifuged at 300g for 6m at 4°C. The supernatant was aspirated from the cell pellet, then the cells were placed in a tube rack, and surrounded in ice.

Annexin-V and Propidium Iodide

Samples were incubated with 10 μ l of 0.1 mg/ml rat IgG to serve as a blocker, and were then washed with PBS. This was followed by aliquotting 10^5 cells each into culture tubes, and 5 μ l Annexin-V were added to each sample. After the Annexin-V incubation, 5 μ l of PI were also added to each tube. Cells were then gently vortexed and allowed to incubate for 15 min at 25°C in the dark. They were then sent to the FACS core facility at UMMS for testing within an hour. Cell gating and data analysis for the samples are examined in the Results section of the report.

Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling (TUNEL)

TUNEL is a late-stage apoptosis assay that detects the presence of DNA fragmentation, one of the hallmarks of late stage apoptosis. DNA strand breaks are labeled with TdT, catalyzing the polymerization of fluorescein-labeled nucleotides to nicked ends. Fluorescein can be detected via fluorescence microscopy as an intermediate check on the assay, and is then bound by anti-fluorescein antibody conjugated with horseradish peroxidase (HRP/POD). After the precipitation reaction, cells can be analyzed via light microscopy. The kit used was the Roche Applied Sciences *In Situ* Cell Death Detection Kit, POD.

Slide Preparation

Cervical lymph node and pancreatic slides were retrieved from the cell morphology core at UMMS and heated to 60°C to remove any lingering wax. Slides were then washed in xylene 3 times, and rehydrated through a 3-minute ethanol bath series (100%, 90%, 80%, 70%, 0%). Cells were then permeabilized in 0.1% Triton X-100, 0.1% Sodium Citrate for 8 min at RT. Slides were blocked in 2 mg/ml BSA + 20% serum in PBS for 30 min, followed by two 1 min rinses in PBS.

Labeling Nicked DNA

Positive controls were treated with 1500 U/ml DNAase I in 50 mM Tris pH 7.5, 10 mM MgCl₂, 1 mg/ml BSA. Slides were then incubated 10 min at RT, and rinsed 3 times in PBS for 5 min each. Label and enzyme were applied to all cells except the

negative controls, which were treated with label solution only. All slides were cover slipped and allowed to incubate at 37°C for 60 min in the dark. Slides were then rinsed in PBS for 5 min at RT, 3 times. Fluorescence microscopy was used at this point as an assay check; the label is conjugated to a protein that is visible via fluorescence microscopy.

Staining Nicked DNA

Converter-POD (Roche) (50 µL) was added to each slide, followed by a 30 min incubation at 37°C. This step conjugated horseradish peroxidase to the fluorescein-labeled nucleotides on the slides. Slides were then rinsed in PBS for 5 min, 4 times each. DAB (substrate for the precipitation reaction) and peroxide buffer (1:10) were used to precipitate the label, effectively staining nicked DNA. Following the precipitation reaction, slides were quickly rinsed in PBS at RT. Counterstaining was performed with Hematoxylin, followed by differentiation solution (Scott's water). Slides were mounted with crystal mount. Results were observed via light microscopy.

Alternate TUNEL Procedure

Slide Preparation

Pancreatic sections were frozen and stored at -70°C until use for the TUNEL assay. No rehydration process was necessary, so the slides were rinsed with Dulbecco's Phosphate-Buffered Solution (PBS) and then fixed in 4% paraformaldehyde (PFA) in PBS for 20 min. Slides were then rinsed in TBS, 3x 10 min. Sections were incubated with DNAase for 10 min in a dilution series as follows (U/mL): 0 (negative control), 100, 250, 500, 1000. Slides were bathed in TBS/T, rinsed once, then washed twice for 10 min

each. Sections were digested with Proteinase K (1 $\mu\text{g}/\text{mL}$) for 30 min at RT, and washed in 2 mg/mL glycine, 2x for 5 min. Slides were re-fixed in 4% PFA in PBS for 20 min. Sections were then washed in TBS/T, 3x for 10 min each wash.

Labeling Nicked DNA

Slides were pre-incubated on ice at 4°C with TdT enzyme and digoxigenin-labeled nucleotides for 1 hr. The solution concentration for this incubation was as follows: 1 μL TdT solution (Roche), 0.5 μL dig-labeled dUTP in 200 μL TUNEL dilution buffer as described in the first TUNEL procedure. Slides were then incubated with enzyme and nucleotides for 1hr at 37°C with enzyme and nucleotides. Sections were rinsed with TBS/T once, and washed in TBS/T 3x for 10 min each wash. Slides were blocked overnight at 4°C in serum and BSA (2mg/mL).

Staining Nicked DNA

Cells were incubated with alkaline-phosphatase-conjugated anti-digoxigenin Fab antibody for 3 hours at a 1:4000 dilution in blocking serum. Cells were washed 3x for 15 min in TBS/T and then left overnight in TBS. Slides were then washed 3x for 15 min in NBT/BCIP staining buffer, freshly prepared and constituted as follows: 0.1M Tris pH 9.5, 0.1 M NaCl, 0.01 M MgCl_2 , 1% v/v Tween-20 (10%), 0.5 mg/mL Levamisol, in 100% water. Cells were then incubated in staining buffer with NBT 2.25 $\mu\text{L}/\text{mL}$ and BCIP 3.5 $\mu\text{L}/\text{mL}$. The reaction was observed after approximately 20 min, and cells were washed in TBS/T 3x for 5 min each to prevent overdevelopment.

Light microscopy was used to observe the precipitate left by the development reaction for positive controls. A combination of DAPI-staining and light microscopy were used to verify the presence of DNA without precipitate in the negative control.

RESULTS

Apoptosis in BB/DP Rat Lymph Nodes

Fluorescence-Activated Cell Sorting was performed on single cell suspensions (in RPMI) of lymph nodes of DR and DP rats. DR rats served as a negative control, and were used to test the experiment as well as to establish the background fluorescence level. Samples were run with and without annexin-V and propidium iodide for DR rats to establish background levels. Experimental samples were taken from two DP rats to verify lymphopenia. DP rats experience profound lymphopenia even before the occurrence of diabetes, so this serves as a control to establish the condition of the experimental rats as pre-determined for diabetes.

The ungated samples contain a significant amount of cells and other material aside from lymphocytes (which include the T-cells of interest in this assay). To analyze the relevant sample content, gates were set to limit the data analysis to lymphocytes in the sample. In particular, the gates were set according to forward scatter (FSC, x-axis) and side scatter (SSC, y-axis) values for the cells. The gates are shown in detail in Figure 7. Elements with high SSC values were characterized as debris, generally too light for lymphocytes. In contrast, elements with high FSC and high SSC were determined to be dead cells and larger aggregates of cellular debris. Elements with high FSC and low SSC values were negligible, at less than one percent of all events analyzed. The area of interest is labeled as the T-cell gate in Figure 7, and has the moderate FSC and low SSC values characteristic of lymphocytes.

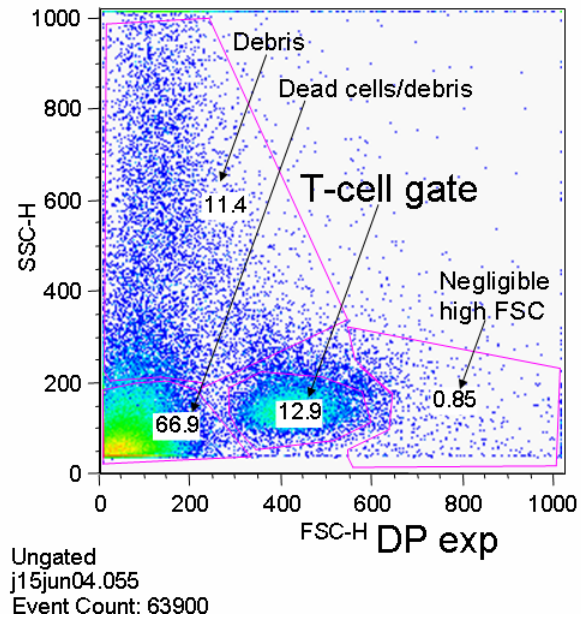


Figure 7: Cell Gating for FACS Analysis. Lymphocytes exhibit a characteristic moderate FSC (X-axis), and low SSC values (Y-axis), which can be seen in the gate marked T-cell gate. Other gates were set around cellular debris and dead cells. Events with high FSC values were declared negligible, rating at less than one percent.

The gates shown in Figure 7 were applied to all samples for uniform analysis, and the resulting gates with regard to each sample are shown in Figure 8, along with the resulting percentages for cells in each gate.

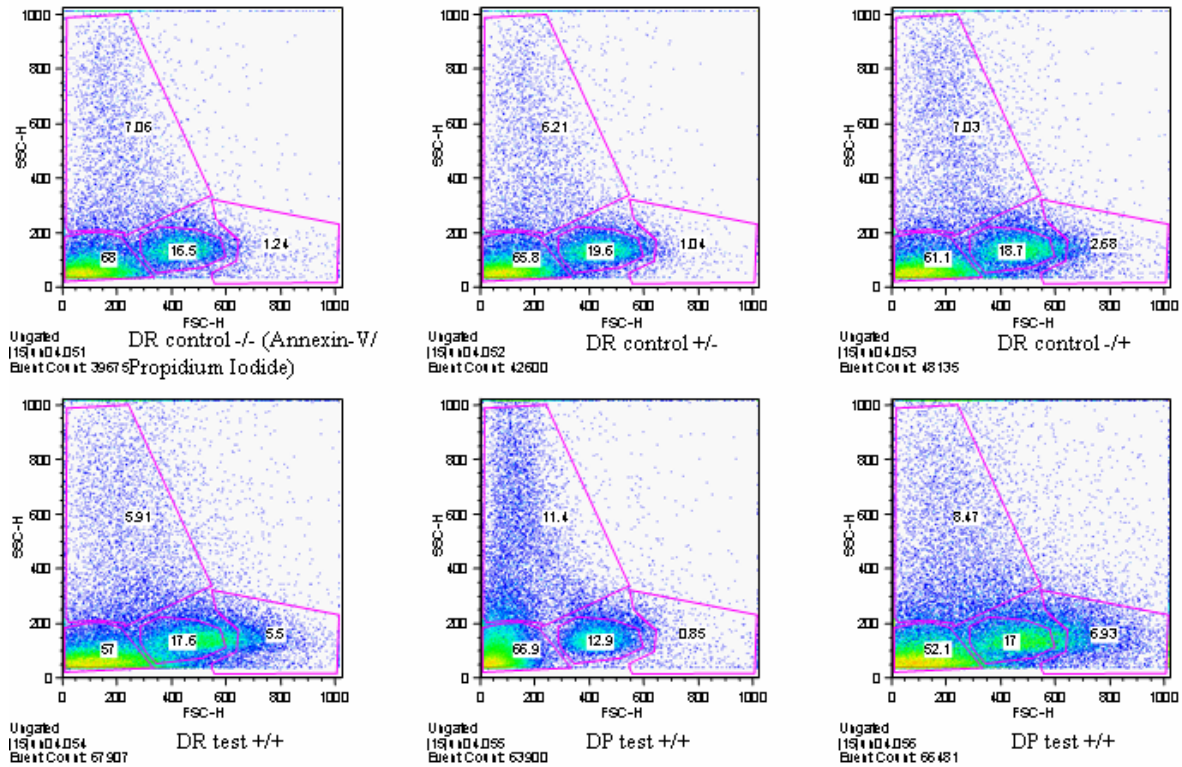


Figure 8: Gates for FACS Analysis of Six Lymph Node Preps. Rat DR and DP lymph nodes were homogenized and suspended in RPMI, and subjected to FACS analysis. Gates shown in Figure 7 were applied to all samples, resulting in the gates shown above.

After establishing the lymphocyte/T-cell gate for all the samples, analysis of the data regarding annexin-V and propidium iodide was possible. Negative controls were used to set the boundaries for establishing the presence of cells binding the two proteins in a manner similar to that described above. As the annexin-V and the PI used were both conjugated to different colors, simultaneous analysis was possible. Figure 9 shows the results of the experiment. Cells binding neither annexin-V (Y-axis) nor PI (X-axis) were determined to be viable; those binding only annexin-V were apoptotic; those binding both annexin-V and PI were declared necrotic.

As shown in Figure 9, the DP samples exhibited a much higher frequency of apoptosis, ranging from 4-7 fold higher than the background level established by the controls (40-73% compared to 8-12% in the controls). This data serves as adequate verification of lymphopenia, and that the rats to be used for experimentation are predisposed to develop diabetes.

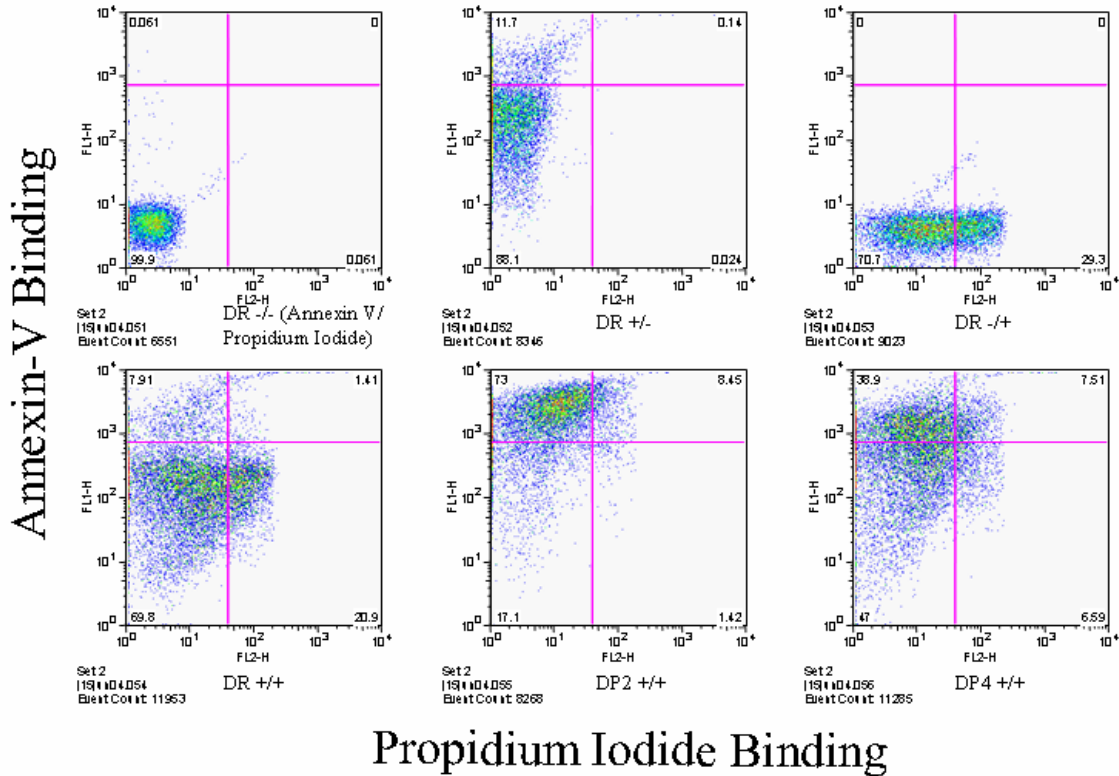


Figure 9: Annexin-V Results for DP and DR Lymph Node Cell Preps. The upper left quadrant indicates cells that are Annexin-V positive and PI negative (apoptotic); the upper right quadrant indicates the intersection of PI positive and Annexin-V positive cells (necrotic); the lower right quadrant shows cells that are PI positive and Annexin-V negative (necrotic/background); and the lower left quadrant shows cells that are negative for both Annexin-V and PI (viable).

Apoptosis in BB/DP Rat Pancreata

After verifying the condition of the rats as diabetes prone, TUNEL was performed on pancreas sections of the rats as described in the first TUNEL procedure listed in Methods. Though the experiment was performed several times using this method, only one set of slides stained correctly for the positive and negative controls. The positive control was treated with DNAase before analysis to ensure the presence of nicked DNA. The negative control was subjected to the proper series of washes and labels, but was not treated with terminal deoxytransferase (TdT). Results were viewed via fluorescence microscopy and light microscopy; pictures of the light microscopy were used to record the results, shown in Figure 10.

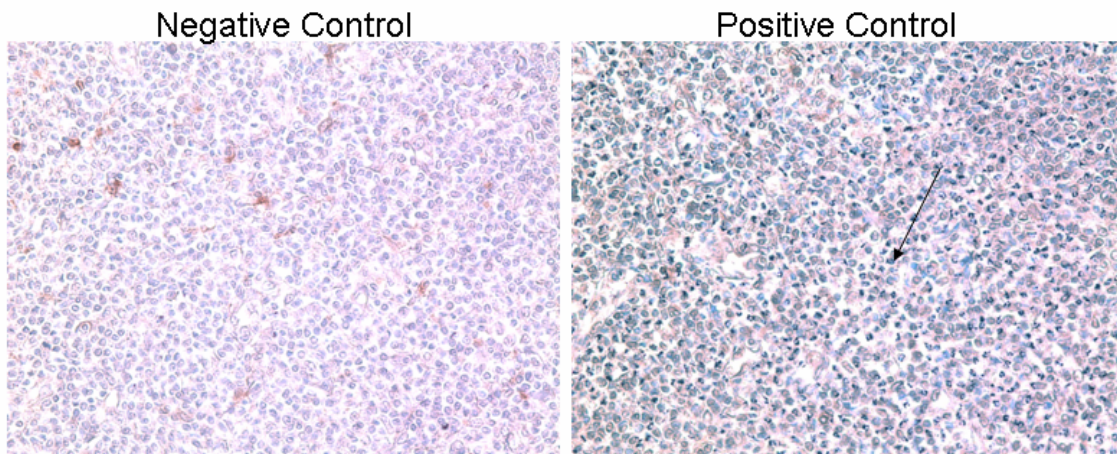


Figure 10: TUNEL Controls for DP Pancreatic Sections. These results were obtained using the first procedure discussed in the Methods section. Nicked DNA is visualized in the positive control as the presence of a green precipitate. This precipitate is not present in the negative control.

Though the TUNEL procedure was successful for this experiment, repeated trials did not yield results, even for the positive controls. As a result, another procedure was adopted for the TUNEL assay, and is outlined in the Methods section as the second TUNEL procedure listed. There are slight differences in the procedures, particularly in the use of buffers mixed in the lab and the use of a different label to detect nicked DNA. The basic principles at work in the procedure, however, remain consistent. Labeled nucleotides bind nicked DNA, and a precipitation reaction is used to visualize the results.

The new procedure was first used on slides in the set used for the first TUNEL attempts, though these slides had not been treated themselves. Treatment of these slides yielded no evidence of nicked DNA, even in positive controls (treated with DNAase as above). Because of these negative results, the presence of DNA was tested by applying DAPI (a nuclear stain) to each section. Although there should have been very specific binding to cell nuclei, all that was observed on any of the slides was a very diffuse blue, indicating that the DAPI was unable to bind DNA.

This development encouraged the use of slides prepared in another fashion; it seemed as though some aspect of the slide preparation process had prevented anything from binding to the DNA on the slides. As binding nicked DNA with labeling proteins is the crux of the TUNEL assay, slides prepared in that fashion were deemed unfit for analysis. To test and optimize positive controls for the TUNEL assay, the procedure was repeated using sections that were frozen and stored at -70°C , without embedding them in paraffin. To ensure that there was accessible DNA on these slides, we first tested a sample slide using DAPI as above. In contrast to the paraffin-embedded slides, highly specific nuclear binding was observed (Figure 11). In addition to one negative control,

slides were run that had been treated with DNAase in the following concentrations (listed in U/mL): 100, 250, 500, and 1000. Although all DNAase treated-samples displayed evidence of nicked DNAase, the slides treated with higher concentrations of the enzyme were over-digested; only the 100U/mL sample was photographed. The results of this optimization experiment are displayed in Figure 11.

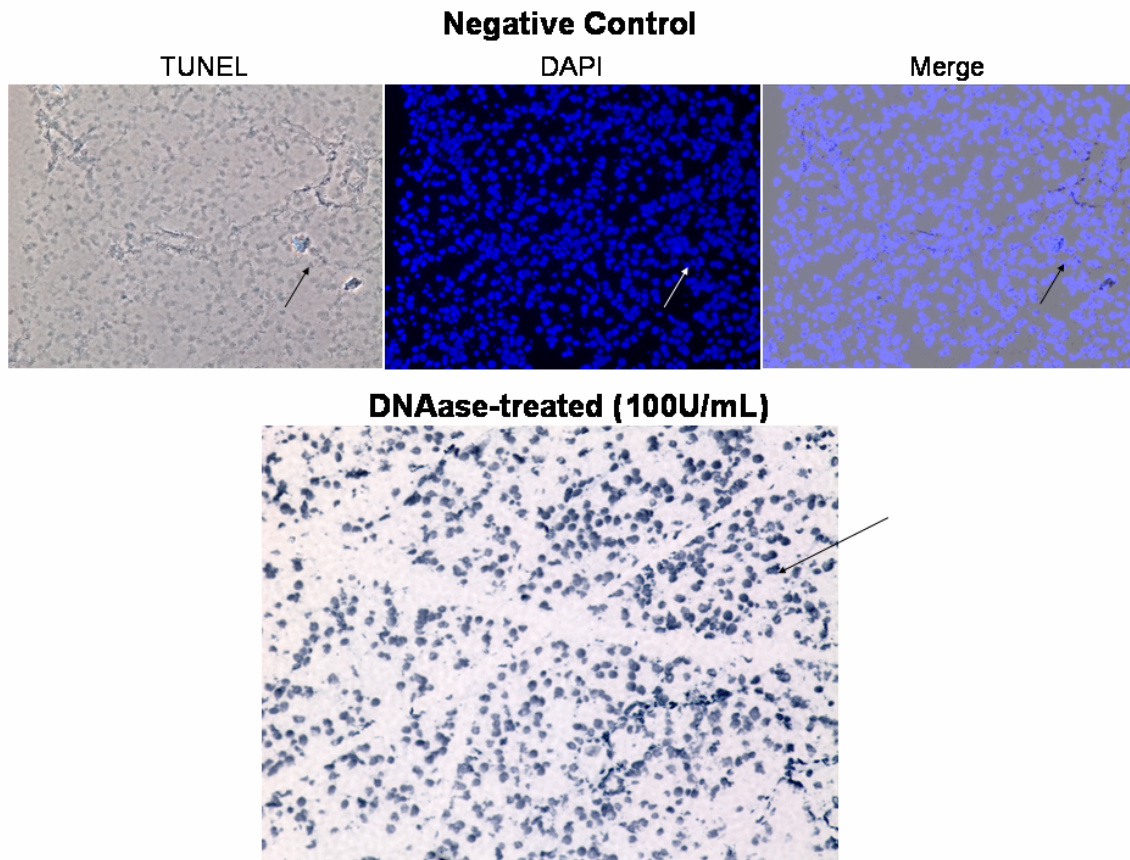


Figure 11: TUNEL Controls for DR Pancreatic Sections. These results were obtained using the second procedure discussed in the Methods section. The negative control (upper row) exhibits no signs of Nicked DNA, visualized in the positive control as the presence of a dark blue precipitate (lower panel). This is checked against a nuclear stain (DAPI), which verifies that there is DNA on the slide. The Merge shows the nuclear stain overlapped with the TUNEL results. Merging was not possible with the DNAase-treated samples; as TUNEL is a precipitation reaction, aggregates of precipitate blocked the DAPI from binding, preventing specific nuclear binding.

DISCUSSION

The first conclusion that can be made from the experimental results is that the DP rats studied were subject to profound lymphopenia. This is supported by the FACS data shown in Figures 7-9 of the Results section. The increased frequency of Annexin-V positive/PI negative cells is indicative of increased numbers of apoptotic cells. As noted in the results section, the amounts of apoptotic cells in the DP experimental rats were 4-7 times more than the background readings established by the DR control rats (DR rats have not been shown to exhibit widespread lymphopenia without provocation). This fact allows characterization of the experimental set of rats (DP) as typical diabetes prone rats, and as possessing a high likelihood of developing diabetes.

After establishing the experiment as a valid test of rats that will become diabetic, it was necessary to investigate the presence of apoptotic β -cells. To this end, a number of TUNEL assays were performed on DP pancreatic sections. Although the assay using the first procedure appeared to be successful once, attempts to recreate data with positive controls were consistently ineffective (Fig. 10). For this reason, a procedure change was made, and several reagents changed. This procedure was also initially ineffective with the slides intended for this project, but yielded strong signals with positive controls when preserving the slides in paraffin was avoided. The fact that frozen sections yielded reliably useful data while paraffin-embedded sections did not, indicates that some step in the de-paraffinization procedure altered the DNA, or that de-waxing was not completely successful in removing the paraffin. If the DNA was damaged in the preservation process, the slides would not be accurate indicators of apoptosis, at least using the

TUNEL assay. Conversely, a thin coating of wax could have prevented proteins from acting on and binding to DNA in the cells, also disabling the TUNEL procedure from indicating the presence of nicked DNA.

The positive controls and the resulting data provide somewhat of a simulation of how positive results would appear in DP pancreata (Fig. 11). The number of cells undergoing apoptosis, however, would be far fewer than appears in the controls. This is due to the fact that in the positive controls, all cells were subjected to DNAase treatment, whereas in the hypothetical DP pancreata, only β -cells would exhibit apoptosis. Even then, only a small number of cells might be required to undergo cell death in order to initiate a larger-scale immune response, resulting in complete destruction of pancreatic islets. As a result, apoptosis would not occur in nearly every cell, as depicted in the positive controls. Rather, β -cells within the islets would undergo apoptosis relatively infrequently.

Because the frozen pancreatic sections yielded positive results, it can be concluded that future research should be conducted using frozen sections, rather than those preserved via the paraffin-embedding procedure. That the assay was successful provides some degree of assurance that the second TUNEL procedure, when performed on frozen sections, should yield good data, whether in confirmation or denial of β -cell apoptosis.

The results of this Major Qualifying Project allow neither confirmation nor denial of β -cell apoptosis in DP rats prior to diabetes pathogenesis. The experimental progress, however, should ease further pursuit of the data in question. In future research addressing the role of apoptosis in diabetes development (with the methods outlined in

this paper), treatment of DNA should be of utmost consideration. This would help to prevent setbacks and aid in conducting faster and more efficient research. Also, a variety of assays can be used to test for apoptosis, and doing so allows for a much more robust data set from which to base mechanistic determinations. As such, the use of FACS to analyze β -cell preps in addition to lymph node suspensions is also advised for further research as to the role of DP β -cell apoptosis in diabetes pathogenesis.

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