Evaluation of the pancreatic islet insulitis in NOD mice treated with antiviral drug

Testing the hypothesized viral etiology in diabetes development using the experimental model of recurrence of disease in the non-obese diabetic mouse model.

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6/12/2013

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Abstract
Type 1 diabetes (T1D) is an autoimmune disease characterized by insulin deficiency due to destruction of the pancreatic β-cells. It has been a known disease for thousands of years, turning from fatal to treatable when the insulin was discovered in the beginning of the 1920s. T1D represents 10 to 15 % of all forms of diabetes and occurs most frequently in children and young adults. Observations report of an increasing incidence worldwide and a decreasing age of onset of the disease. The etiological agent responsible for the development of T1D still remains unknown. Some evidence strongly indicates the influence of environmental factors, where virus is a potential candidate. Studies from the bank voles have given the interest to a member of the Picornaviridae, namely the Ljungan virus. Since studies of viruses in the development of T1D in humans are difficult to implement in rodents, especially the non-obese mouse (NOD) and the Bio-breeding rat, are highly valued animal models in this field of research.

In this study we evaluate the degree of pancreatic islet cell mass destruction in transplanted female NOD mice treated with antiviral drug. We have concluded that the prolonged survival time is not a result of resident islet mass at time of transplantation. Thus the state of normoglycemia seen after transplantation is the result of normal function in the islet graft from healthy male NOD donors. Also the increased survival time of the graft is a result of the antiviral treatment.

Sammanfattning

Background
The inflammatory process is important in the development of autoimmune type 1 diabetes. It has been suggested that virus may be involved in the etiology of the disease. Can prevention of viral infection reduce the incidence of type 1 diabetes.

History
Diabetes has been a known human disease for thousands of years. The first symptoms described are written on Egyptian papyri about 1500 BCE. Diabetes was for a long time a fatal disease. Patients were suffering from unquenchable thirst, excessive urination, hunger or lack of appetite, gangrene and a vulnerability to infections. In the end the patients fell into coma and shortly thereafter death followed. The first pathological origin of diabetes was discovered in 1674 as the British scientist Thomas Willis observed a taste of sugar in the blood and the urine of patient suffering from diabetes. He then suggested that diabetes was a disease of the blood and not the kidneys that earlier has been presented. For about 100 years later this theory was confirmed experimentally by Matthew Dobson and diabetes was given the name mellitus, from the Latin word for honey. In 1869 focus directed towards the pancreas when Paul Langerhans described the findings of ductless cells within the exocrine pancreas. He named the cells “islets of Langerhans” and presented a theory that the pancreas had an earlier unknown endocrine function. Not long after did Oscar Minkowski’s trials, which comprised removal of the pancreas from dogs, resulted in diabetes and thereby he could confirm the endocrine function (Ajanki, 2008; Sanders, 2002).

In January 1922 fourteen-year-old Leonard Thompson, dying because of his diabetes, was given the first insulin treatment (Allan, 1971). This was a result of experiments that had started almost a year earlier in JJR Macleod’s laboratory at the University of Toronto. Frederick Banting, and his assistant Charles Herbert Best, experiments on dogs finally resulted in the discovery and the isolation of insulin, which is one of the greatest findings in the diabetes history. Diabetes had now turned from a fatal to a treatable disease and in 1923 Banting and Macleod were awarded the Nobel Prize in Medicine, which they shared with their two assistants Best and a biochemist named James Bertram Collip. Later; the Nobel Prize has also been awarded to Frederick Sanger in 1958 for describing the structure of proteins of which insulin was one of the selected and to Rosalyn Sussman Yalow in 1977 for the development of the radioimmunological assay used in the detection and quantification of insulin in samples (Sanders, 2002).
It soon became clear that insulin did not cure diabetes. Serious secondary complications, including blindness, kidney failure, cardiovascular disease and amputations were seen in insulin-treated patients (Sanders, 2002). The understanding of the disease as well as the development of techniques to facilitate the control of blood glucose levels have been highly valued during the years. But still the biggest questions remain: why does diabetes occur and will there be a possible cure?

In 1965 Gepts (Gepts, 1965) presented the first results that would lead the research toward the modern era of the diabetes research. He had observed an inflammatory process, called insulinitis, in pancreatic islets from diabetes patients. When islet antibodies were discovered, about 10 years later, diabetes became categorized as an autoimmune disease (Bottazzo et al., 1974). Since then big focus has been directed against the components of the immune system and possible factors that could trigger an autoimmune response.

**Diabetes**

Type 1 diabetes (T1D) is one of the two major clinical forms of diabetes. It is classified as an autoimmune disease, where destruction of the pancreatic β-cells lead to insulin deficiency and loss of function to regulate the blood glucose. T1D represents 10 to 15 % of all forms of diabetes and is most frequently occurring in children younger than 15-18 years. Observations report that the incidence has been increasing approximately 2-5% per year worldwide and that there is a decreasing age of onset of disease, particularly in children younger than 5 years (Daneman, 2006). There are large geographical variations, where the north part of Europe, but also Sardinia, has the highest incidence of T1D (Soltesz et al., 2007).

The etiological agent responsible for the development of T1D remains unknown. In the 1980s Eisenbarth (Eisenbarth, 1986) suggested that everybody are born with a degree of susceptibility to develop T1D. The strongest genetic factor accounting for about 50 % is the major histocompatibility complex (MHC) class II alleles (Boitard, 2012; Daneman, 2006). However, the low concordance rate in monozygotic twins, which is only 30-40 % and the unexplained incidence rise, indicates of influence from environmental factors. Some potential environmental triggers that have been examined this far are environmental toxins, dietary components as milk-and gluten-proteins, childhood vaccinations and viruses (Daneman, 2006).
Type 2 diabetes

The most common form of diabetes is the type 2 diabetes (T2D). T2D is strongly associated with genetics and life style factors. It is possible that changes in life style is a contributing factor to the global increase in T2D since there is also a global trend in increased obesity and physical inactivity (Olokoba et al., 2012). The majority of T2D is seen in older people, but there is an emerging epidemic in young people (Rosenbloom et al., 1999). T2D is considered a metabolic disease with an insufficient insulin signaling that result in an increased need of insulin production (Olokoba et al., 2012). The increased demand for insulin can be due to dysfunction of the pancreatic β-cells (Marchetti et al., 2010) or to systemic insulin resistance. Observations like an inflammatory state in the adipose tissue, that may contribute to the insulin resistance (Wellen and Hotamisligil, 2003), and also activation of the immune response in these patients poses the question if T1D and T2D may be more similar than initially thought (Boitard et al., 2005). The large number of people living with the chronic disease diabetes, which implies both short-term and long-term complications, the need for a novel prevention is of great importance.

Pancreatic islet architecture

The pancreas is a mix of both an exocrine gland that produces digestive enzymes and an endocrine gland that produces hormones. The hormones are synthesized in the islet of Langerhans which represent 1-2% of the total volume of the organ. Each islet is approximately 100-200 µm in diameter and contains four types of cells: α-cells secrete glucagon, β-cells secrete insulin, δ-cells secrete somatostatin and PP-cells secrete pancreatic polypeptide (Mescher, 2010). Due to different embryological origin parts of the pancreas consists of different sets of cells. The body and tail consist approximately of 82 % β-cells, 13 % α-cells, 4 % δ-cells and 1 % PP-cells, while the posterior part consists of 79 % PP-cells. In T1D the β-cells get destructed and after that approximately 80 % have been destroyed symptoms of disease occurs (Foulis, 1993).

Pathogenesis

T1D is characterized by an autoimmune-mediated destruction of the insulin-secreting β cells of the pancreas. Indications that T1D is an autoimmune disease relies on the detection of insulitis, islet autoantibodies, β-cell-reactive T-cells and the higher frequency of diabetes in combination with a restricted set of MHC class II alleles. What is triggering the destructive response is today unknown, but animal studies, especially in the non-obese diabetic (NOD)
mouse and the bio breeding (BB) rat, have greatly promoted the understanding of the pathogenesis in human T1D (Boitard, 2012; Yoon and Jun, 2005).

**Insulitis**
Insulitis is an inflammatory infiltration in the islets of Langerhans. The infiltrate is dominated by CD8+ T cells, but also consists of CD4+ T cells, B cells and macrophages (Willcox et al., 2009). Insulitis is known to be a part of the pathogenesis in animal models developing autoimmune T1D, as the NOD mouse and the BB rat (Kolb et al., 1996; Zipris, 1996). Human data shows various results. There is a limited amount of material available to study and most of it is post-mortem. Material from a fulminant version of the disease may not represent the characteristics of the disease process (In't Veld, 2011).

**Autoantibodies**
An autoantibody is an antibody produced by the immune system and directed to endogenous epitopes. The immune mediated destruction of β-cells is usually preceded by the development of autoantibodies to pancreatic β-cell autoantigens (Taplin and Barker, 2008). This development is associated to a high-risk HLA haplotype and occurs months or years before onset of disease but may be presen as early as first year of life (Barker et al., 2004). The autoantibodies include islet cell antibodies (ICA), antibodies to insulin (IAA), glutamic acid decarboxylase (GAD) and protein tyrosine phosphatase (IA2) (Taplin and Barker, 2008). Autoantibodies are not believed to be involved in pathogenic events, but are good as prognostic markers. Two or more autoantibodies give a higher risk for developing T1D than if just single autoantibodies are presented (Yoon and Jun, 2005).

**Autoimmunity**
T1D is an autoimmune disease, which is an immune reaction against endogenous antigens so-called autoimmunity. An autoimmune reactions starts with the presentation of an antigen in the MHC class II locus, which is located on the surface of an antigen presenting cell (APC). The complex of the antigen and the MHC class II loci is specifically recognized by specific cells from the adaptive immune system named CD4+ T cells. The CD4+ T cell, can dependently of the type of antigen, the type of the APC and the MHC class II haplotype differentiate into two specific T helper subset: Th1 and Th2 (Liblau et al., 1995). Th1 cells produces cytokines and are associated with cellular immunity while Th2 cells are associated with humoral immunity. Autoimmune reactions are a normal part of the immune system but an imbalance between the two T helper subsets may promote disease (Scott, 1993).
dominant Th1 response has been associated with T1D, while Th2 has been associated with protection (Boitard, 2012). In T1D different mechanisms leads to a final stage of the autoimmune response where the β-cells are getting destroyed (Buschard, 2011).

**Destruction of the pancreatic β-cells**

The autoimmune-mediated destruction of the β-cells starts when CD8+ T-cells and macrophages become cytotoxic by the activation from the Th1 cells. Cytokines that are directly cytotoxic to the β-cells are secreted both from the Th1 cells and the macrophages, which also produces oxygen free radicals. Cytokines released from macrophages may also have a role in maximizing the effect of CD8+ cytotoxic T cells. The CD8+ cytotoxic T cells are specialized to recognize specific antigens on the β-cells through the association with the MHC class I molecule. Destruction of the β-cells then follows either through direct contact with apoptosisinducing receptors on the cell surface, or by secretion of protease granzymes that will activate nucleases in the cell and thereby kill the β-cells (Yoon and Jun, 2005).

**Virus in type 1 diabetes**

As an environmental factor virus is strongly associated with the development of T1D (Jun and Yoon, 2003). Observations that diabetes sometimes followed acute infections such as mumps were reported already in the late 1800’s (Harris, 1898). Early observations also reported that diabetes occurred more frequent at certain times of the year (Adams, 1926) which may indicate on a viral cause. Later has virus-specific antibodies been presented in patients with T1D (King et al., 1983). Viruses have also been found in the pancreas of patients that died from acute T1D. Some of these have been isolated and shown to cause diabetes in animals (Yoon et al., 1979).

Until now a numerous of viruses have been examined in the association to T1D. It is suggested that the pathogenesis of virus-caused T1D can be divided into at least two ways. First, the viruses may directly infect and destroy the β-cells. Second, the viruses may cause an autoimmune attack on the β-cells by either infect the β-cells or by a systemic infection. An infected β-cell can be triggering an autoimmune response by expressing viral antigens, expressing altered β-cell antigens or expressing molecules as cytokines. A systemic response may alter the immune system of the host. These alterations could include activation of B-cells, leading to production of autoantibodies, or a disruption of the Th1/Th2 balance. Also, the epitopes of the viruses could be similar to the β-cells which could generate CD8+ cytotoxic T cells that incorrectly target the β-cells (Jun and Yoon, 2003).
Even if there are studies that show correlation between viral infections and T1D, there are also studies that have not found any evidence of the involvement of viruses. It is however possible that the lack of correlation depends on the numerous genetic variations of viruses and that the immune response differs among individuals. Studies of viruses in the development of T1D in human are difficult to implement and today the cause remains to be established (Jun and Yoon, 2003).

**Ljungan virus**

After reports of a co-variation of the incidence of T1D in humans with the three- to four-year population density cycle of the bank voles in the north part of Sweden (Niklasson et al., 1998) it has also been shown that bank voles in Denmark and Sweden develops diabetes. The symptoms are similar to human T1D with glucosuria, hyperglycemia, a gradually developing lethal ketoacidosis and also the present of autoantibodies as GAD65, IA-2 and IAA could be found. The pancreatic tissue showed total destruction in end-stage animals (Niklasson et al., 2003b). Interestingly a picornavirus, namely the Ljungan virus could be isolated and detected in the islet cells of the bank voles with diabetes (Johansson et al., 2003; Johansson et al., 2002; Niklasson et al., 2003a; Niklasson et al., 1999) and according to Niklasson et al(Niklasson et al., 2003b), the Ljungan virus is a likely etiologic agent of type 1 diabetes in bank voles. Ljungan virus is classified as a member of the genera Parechovirus of Picornaviridae. Picornaviruses are plus stranded RNA viruses, where also Coxsackievirus, human infectious agent of Enterovirus genus, is a member (Gamble et al., 1969). Especially Coxsackie B4 has been suggested to be a causative factor to human T1D (Barrett-Connor, 1985; Gamble et al., 1969; Gamble and Taylor, 1969; Szopa et al., 1993).

**Animal models**

Animal models of diabetes have been used extensively ever since the early days of diabetes research (Rees and Alcolado, 2005). Today, the rodents play an important role in the investigation of the disease mechanisms and in the development of possible treatments. The non-obese diabetic (NOD) mouse and bio breeding (BB) rat, which spontaneously develops T1D are the most common animal models used (Bortell and Yang, 2012; Kachapati et al., 2012; Rees and Alcolado, 2005). It is notable that these animals are highly inbred and therefore when discussing human pathophysiology of diabetes, caution to its relevance must be considered (Rees and Alcolado, 2005).
The Bio Breeding Rat
The BB rat is a result from inbreeding of selected individuals from the Wistar rat colony performed in the 1970s at the Bio-Breeding Laboratories in Canada. (Like et al., 1991; Rees and Alcolado, 2005). This rat strain has become an important model for studying spontaneous developing T1D and especially the role of environmental factors, since certain treatments result in the development of the disease (Bortell and Yang, 2012; Mordes et al., 2004). The Diabetes-Prone BB (BBDP) rat develops T1D spontaneously, while the Diabetes-Resistant BB (BBDR) variant does not develop diabetes in viral antibody-free conditions (Like et al., 1991). As with humans, genes of the MHC class II provides susceptibility to develop autoimmune T1D (Colle et al., 1990; Fuks et al., 1990). Insulitis, morphologically similar to insulitis observed in humans, can also be seen in the BB rat (Kolb et al., 1996; Zipris, 1996).

The Non-obese Diabetic Mouse
The NOD mouse is a widely used model for understanding of the human T1D and to prevent, delay and to identify potential treatments for the disease (Atkinson and Leiter, 1999). The NOD mouse developed in 1974 after selective breeding from a female mouse from the JCL-ICR strain. It was used in studies for cataract when it spontaneously showed diabetic symptoms (Makino et al., 1980). Diabetes in NOD mouse is both complex and multifactorial (Delovitch and Singh, 1997; Rabinovitch, 1998). The MHC class II alleles of the NOD mouse are a major contributing genetic component of susceptibility to T1D (Serreze et al., 1997). Destruction of the β cells in the pancreatic islets is preceded by early events consisting of presentation of islet antigens in the pancreatic lymph nodes (Hoglund et al., 1999) followed by insulitis. The insulitis entails a gradual infiltration of APCs (Rosmalen et al., 1997) which present autoantigen to the CD4+ T cells (Saxena et al., 2007). CD4+ T cells activates CD8+ T cells, which mediates a final attack that will lead to destruction of the β-cells (Wong and Janeway, 1997). Onset of disease is present between 12 and 30 week of age (Rees and Alcolado, 2005). There is a gender difference with an incidence of 60-80% in female and about 10% in males (Makino et al., 1980).
Project focus

This project investigates the possible effect of antiviral drug treatment on diabetes development, studying the recurrence of disease (ROD) in the NOD mouse model. The project focuses on testing the hypothesis that the NOD mouse model may be a virus-induced diabetes animal model.

Aim of the study

The aim of this study was to confirm immune-mediated destruction of the islet cell mass in the pancreas by characterizing the inflammatory state of the islets in antiviral drug treated female diabetic NOD mice made normoglycemic with islet graft from healthy male NOD donors.

The tissue samples studied were examined in bright field microscope and evaluated using the islet insulitis scoring method in order to:

- Investigate the presence or absence of healthy pancreatic islets in antiviral drug treated transplanted animals.
- Determine the pancreatic islet insulitis progression in antiviral drug treated transplanted animals.
- Study the possible correlation between the pancreatic islet insulitis score and the survival time of the antiviral drug treated transplanted animals.

Fixation

To save and preserve the structure and the chemical composition of the tissue, it is necessary that a fixation is implemented (Kiernan, 2008). One of the most commonly used fixative agents in light microscopy is neutral buffered formalin (Gartner, 2001). Formalin is a solution of formaldehyde in water that has been buffered to pH 7.2-7.4. Formaldehyde, which is the active molecule, reacts with several parts of protein molecules. These reactions may inhibit enzymes and thereby prevent autolysis. Further reactions may also lead to that different proteins will be joined together and that such constitutions will provide a structural stabilization of the tissue. It is notable that fixatives can affect the tissue properties and in particular it hardens the tissue (Kiernan, 2008).
**Embedding**

To facilitate the cutting of the tissue into thin sections the preparations get embedded in a hard medium. A commonly used embedding medium for light microscopy is paraffin. In order to get the best result during embedding the tissue must first be dehydrated. To remove the water from the tissue, a series of alcohol bath, starting with 70% and progress in graded steps to 100%, is used. A final step is needed where the tissue is treated with a chemical that is miscible with paraffin, for example xylene. After treatment with this lipophilic solvent the tissue can be put in a paraffin bath and be completely perfused. When the paraffin is hardened it forms a block, suitable for the sectioning of the tissue (Gartner, 2001; Young, 2006). In addition to the fixation, also the embedding affects the tissue. According to J.A Kiernan (Kiernan, 2008) a fixed, paraffin-embedded tissue is commonly 60-70% left in size of what it was in life.

**Sectioning**

Sectioning of the tissue is necessary for the microscopical examination. The thickness of the sections need to be thin enough for being transparent but still maintain contrast that enables resolution of structural details. Paraffin-embedded tissue can be sectioned by using a microtome (Kiernan, 2008).

**Staining**

In order to see the structures in the tissues the preparations needs to be stained. Hematoxylin and eosin staining was from the beginning used in the textile industries and is today the most commonly used method in histochemistry laboratories (Avwioro, 2011; Mescher, 2010). The primary function of this staining method is to clarify structural characteristic at the preparations (Ross, 2003), by distinguish the differences between acidophilic and basophilic materials (Avwioro, 2011). Hematoxylin and eosin is a combination of a basic and acid dye. Hematoxylin, which is basic, stains acidic structures, such as DNA and RNA-rich portions blue. The acidic dye eosin stains basic structures, as many of the cytoplasmic components and collagen, pink (Mescher, 2010; Young, 2006).

Since the Hematoxylin and eosin stain is an aqueous solution the tissue preparations must first be placed in a clearing agent, for example xylene, to dissolve and remove the paraffin. That is
followed by a passage through a series of alcohol, graded from 95-70%, which step by step will hydrate the tissue (Kiernan, 2008).

**Islet evaluation**
The histology of the pancreatic islets can be evaluated by using a bright field microscopy. The insulitis can be scored in four levels. The total amount and the size of the islets in the preparations is taken into account together with the infiltration of lymphocytes. The scoring is classified by; A: normal islet, B: infiltration of lymphocytes in the peri-insular area, C: infiltration of lymphocytes in the islet, D: Small and few islets with disturbed architecture.

*Figure 1.* Illustration of the islet evaluation; A: normal islet, B: infiltration of lymphocytes in the peri-insular area, C: infiltration of lymphocytes in the islet, D: Small and few islets with disturbed architecture. The scale bar equals 100 µm.
Materials and Methods

Animal and islet preparations
Animals were kept under standard conditions according to protocols of the laboratory animal facility at BMC, Uppsala University. The animals had free access to water and pelleted standard food for rodents. The experimental procedure has been approved by the Uppsala Ethical Committee in Uppsala (133/11) and is in accordance with international guidelines (NIH publications no.85-23, revised 1985).

Pancreatic islets were isolated from healthy male NOD mouse individuals 8-10 weeks of age. The pancreatic islets were isolated according to a collagenase degradation and braking pipette isolation procedure, described previously (Blixt et al., 2007). The isolated islets were cultured in 3 – 6 days under standard islet culturing conditions described previously (Andersson, 1978). Female NOD mice were regularly screened for glucosuria using Diastix reagent strips (Bayer, Germany). The blood glucose concentration was measured in animals that were positive for glucosuria, using a sample taken in the capillary vein-blood from the tail tip and the FreeStyle Lite blood glucose meter (Abbott, Illinois, USA). Animals with a blood glucose level above 14 mM glucose were considered to qualify to enter the experiment. The female diabetic NOD mice that enter the project were made normoglycemic with islet graft that was previously isolated from healthy male NOD donors. The islets were placed under the renal capsule where high blood flow enables good oxygen levels.

Antiviral drug treatment
The antiviral drug treatment was started 24 hours before transplantation of the pancreatic islet graft using a gavage feeding procedure. Transplanted animals were then gavage fed with an antiviral substance two times per day and their blood glucose was tested regularly each morning.

Experimental setup
Female diabetic NOD mice made normoglycemic with islet graft from healthy male NOD donors are here by referred to as transplanted animals.

- The transplanted animals were gavage fed with vehicle or with the antiviral drugs Pleconaril and Ribavirin in combination. The treatment started one day prior to the transplantation procedure and then succeeded continuously twice a day until euthanization.
• The development of ROD in the experimental animal was set as one endpoint of the experiment. The ROD was defined as elevated blood glucose concentration above 11.1 mM on two consecutive days.

• The second endpoint was defined as the euthanization of animals that displayed reduced health status according to the Hampshire healthcare scoring chart but without developing ROD (Hampshire et al., 2001). There were no animals found diseased during the experiment.

**Tissue preparations**

Pancreatic tissues were collected by dissection from all the animals in the trial. Fixations of the tissues were then done by using 10 % formalin for 24 hours. To avoid microbial growth, that may lead to destruction of the tissues, and to prepare for the embedding of the tissues the samples were dehydrated. The dehydration was done in a Microm STP 120 (Waltham, Massachusetts, USA) according to the following schedule:

<table>
<thead>
<tr>
<th>Liquide</th>
<th>Temperature</th>
<th>Time</th>
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<tbody>
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<td>RT</td>
<td>Stored</td>
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<tr>
<td>80% EtOH</td>
<td>RT</td>
<td>60 min</td>
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<tr>
<td>80% EtOH</td>
<td>RT</td>
<td>60 min</td>
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<tr>
<td>95% EtOH</td>
<td>RT</td>
<td>50 min</td>
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<tr>
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<td>RT</td>
<td>50 min</td>
</tr>
<tr>
<td>Abs</td>
<td>RT</td>
<td>45 min</td>
</tr>
<tr>
<td>Abs</td>
<td>RT</td>
<td>45 min</td>
</tr>
<tr>
<td>Abs/Xylene</td>
<td>RT</td>
<td>30 min</td>
</tr>
<tr>
<td>Xylene</td>
<td>RT</td>
<td>10 min</td>
</tr>
<tr>
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<td>RT</td>
<td>10 min</td>
</tr>
<tr>
<td>Paraffin</td>
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</tr>
<tr>
<td>Paraffin</td>
<td>60°C</td>
<td>120 min</td>
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</table>

The tissues were then embedded in small blocks of paraffin, which were sectioned by using a Rotary Microtome HM 355 S (Walldorf, Germany). In this experiment, where pancreatic tissues are examined in bright-field microscopy, it has been proven that 5 µm thin sections are to be preferable.
To get an overall picture over the whole pancreatic tissue the blocks were divided into three levels. In the first level 135 sections were collected, then the following 40 sections were discarded, after which another 135 sections were collected and this constituted the second level. The same procedure was made for the third level. 405 sections per pancreatic tissue were collected and placed on glass slides – three sections on each slide. Those were then dried in 38 degrees Celsius for at least 3 hours.

The first and the last slide for all of the three levels were then picked for staining. That entailed a collection of 6 slides per pancreatic tissue and a total collection of 84 slides. Before staining, paraffin had to be removed from the tissue preparations, which was done by using xylene. The staining method used was hematoxylin and eosin staining. Because the staining-medium is solved in an aqueous solution all the preparations were hydrated before staining. The slides were then assembled with coverslips by using glue based on xylene. The mounting was made by Leica CV 5030 (Wetzlar, Germany).

**Evaluation**

Evaluation was done by a double-blinded observer, using a bright field microscope, magnification x100. Each slide was observed and the islets scored according to insulitis level. The amount and the size of the islets were taken into account together with the infiltration of lymphocytes. Observations were then taken together and each of the pancreatic tissues were scored as either A, B, C or D (shown in Fig. 1). A: normal islet, B: infiltration of lymphocytes in the peri-insular area, C: infiltration of lymphocytes in the islet, D: Small and few islets with disturbed architecture.

**Statistical analysis**

The mouse islet insulitis scoring was studied on at least three separate sections take a minimum of 200µm apart. Each scored animal was considered one separate observation. Values are expressed as box plot with the 50% (median), 25% and 75% percentiles and 10% and 90% percentiles marked with whiskers. Groups of data were compared using the Fisher’s exact test. Additional test used to test survival time between the groups was the Mann-Whitney rank sum test. Statistical analysis was performed using SigmaPlott (SPSS Inc, Chicago, Il, USA).
Results

Table 1. Treatment and scoring in the experimental groups.

<table>
<thead>
<tr>
<th>Group</th>
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<th>Treatment</th>
<th>Recurrence of disease</th>
<th>Animal islets scoring</th>
<th>Observations</th>
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<td>C</td>
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<tr>
<td></td>
<td>B</td>
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<td>D</td>
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<td>C</td>
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<td>D</td>
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<td>C</td>
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<td>Yes</td>
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<td>I</td>
<td>Plec + Rib</td>
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<tr>
<td></td>
<td>P</td>
<td>Plec + Rib</td>
<td>No</td>
<td>C</td>
<td>A-scored islet observed</td>
</tr>
</tbody>
</table>

Female diabetic NOD mice made normoglycemic with islet graft from healthy male donors were gavage fed with the antiviral drugs Pleconaril and Ribavirin (Plec + Rib) in combination or vehicle, one day prior to transplantation and twice every day until euthanization. Elevated blood glucose concentration above 11.1 mM glucose in these animals measured on two days in a row was considered as ROD. Animals with ROD and animals displaying reduced health status according to the Hampshire model were euthanized. Animals in each experimental group are scored according to the different insulitis categories. A: normal islet, B: infiltration of lymphocytes in the peri-insular area, C: infiltration of lymphocytes in the islet, D: Small and few islets with disturbed architecture. Occasional islets with an aberrant scoring were observed in some animals.

The experimental groups are shown in table 1. Female diabetic NOD mice made normoglycemic, in group I and II the animals were gavage fed with vehicle and in group III and IV the animals were gavage fed with the antiviral drugs Pleconaril and Ribavirin in combination. In the vehicle treated group four out of seven animals developed ROD, defined as elevated blood glucose concentration above 11.1 mM on two consecutive days. In the
antiviral drug treated group five out of nine animals developed ROD. The animal islet scoring shows results of the structural examination. Out of eleven animals there was none scored as A or B, suggesting that there may be no or very little remaining β-cell mass in the pancreatic islets. In group I to III no obvious difference between the numbers of animals in the insulitis categories could be detected. However there is no D scored animals in the group number IV.

In a separate set of experiments the short time effect of the gavage feeding procedure were tested on three transplanted female NOD mice. The animals were gavage fed with saline according to the same protocol used for the experimental animals. There were no differences observed in short time survival when comparing with the vehicle treated group (data not shown).

Table 2. Fisher’s exact test table for the insulitis scoring level and the treatment.

<table>
<thead>
<tr>
<th>Score</th>
<th>Vehicle</th>
<th>Plec + Rib</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>D</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

Fisher’s exact test table testing the possible effect of the treatment on the insulitis scoring in female diabetic NOD mice made normoglycemic with islet graft. The treatments tested are vehicle versus Pleconaril and Ribavirin in combination. The insulitis levels tested are the C and D scoring.

The insulitis scoring level in relation to the possible effect of the treatment are tested in table 2. There were three animals scored as C in the vehicle treated group and four animals in the Plec + Rib treated group. The more severe D scoring was seen in three animals in the vehicle treated group but only in one of the Plec + Rib treated animals. No statistical significance was seen between the treatment and the scoring.
Table 3. Fisher’s exact test table for the insulitis scoring level and the recurrence of disease.

<table>
<thead>
<tr>
<th>Score</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>5</td>
<td>4</td>
</tr>
<tr>
<td>D</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

Fisher’s exact test table testing if female diabetic NOD mice made normoglycemic with islet graft if the insulitis scoring level are different between the animals developing ROD or not. ROD is defined as blood glucose concentration above 11.1 mM on two consecutive days. The insulitis levels tested are the C and D.

In table 3 the insulitis scoring level is examined in the association to the development of ROD or not. In the group of mice that develop ROD there were five C and three D scored animals. However; in the group of mice not developing ROD there were four C and but only one D scored animal. No statistical difference was observed between the groups.

Table 4. Fisher’s exact test table for the effect of the treatment and the recurrence of disease.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Yes</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>Plec + Rib</td>
<td>5</td>
<td>4</td>
</tr>
</tbody>
</table>

Fisher’s exact test table testing if female diabetic NOD mice made normoglycemic with islet graft are developing ROD or not in relation to the treatment. The treatments tested are vehicle versus Pleconaril and Ribavirin (Plec + Rib) in combination. ROD is defined as blood glucose concentration above 11.1 mM on two consecutive days.

The antiviral treatment of Plec + Rib will not delay the ROD indefinitely as shown in table 4. No obvious differences were observed in the two groups of treated animals. Five out of nine animals in the Plec + Rib treated group developed ROD corresponding to four out of seven animals in the vehicle treated group.
Figure 2. The association between the insulitis scoring C or D and survival time.

A

B

Female diabetic NOD mice made normoglycemic with islet graft and treated with vehicle or the antiviral drugs Plec + Rib in combination. Survival period was defined as the number of days after transplantation until euthanization. ROD is defined as blood glucose concentration above 11.1 mM on two consecutive days. In A all animals are taken into account regardless ROD or not. In B only animals developing ROD are presented.

To investigate if the scoring or the treatment had any significance in survival time after transplantation data are presented as boxplots. The distribution of the number of observations is illustrated as a box with whiskers (Fig. 2 and 3). The boxes represent the 50% (median), 25% and 75% percentiles and the whiskers 10% and 90% percentiles. Data are tested with Mann-Whitney rank sum test. In figure 2A and 3A all animals, regardless whether they developed ROD or not, were taken into account and in figure 2B and 3B only data of animals that developed ROD are presented. In figure 2 data of the two scoring levels (C and D) against survival time in days are presented. There were no significant differences between these groups. In Figure 3 data compares the effect of the treatment in relation to survival time. A significant difference was observed in the animals developing ROD between the differently treated groups. This may indicate a possible effect of the antiviral drug to prevent the disease progression.
Figure 3. The effect of the treatment in relation to survival time.

Female diabetic NOD mice made normoglycemic with islet graft and treated with vehicle or the antiviral drugs Plec + Rib in combination. Survival period was defined as the number of days after transplantation until euthanization. ROD is defined as blood glucose concentration above 11.1 mM on two consecutive days. In A all animals are taken into account regardless ROD or not. In B only animals developing ROD are presented. Data are compared using the Mann-Whitney rank sum test; * denotes p ≤ 0.05.
Discussion

The main finding in this study, that the inflammatory process in the islets is severe with no intact islet cell mass, indicates that there was no remaining function of the pancreatic tissue in the animals upon euthanization.

Earlier studies have shown that insulin treatment may support so called “honeymoon phase” or remission of the disease and this makes it very important to confirm that the produced insulin only originates from the islet grafts in our study. If there is functional islet mass in the pancreas at the time of transplantation this may lead to prolonged survival time of the animal. This possible scenario will then be a consequence of increased functional islet mass and not a protective effect of the antiviral drug treatment. However, it is also possible that any protective effect of the antiviral drug may support the survival of resident pancreatic islet mass. The morphological examination did not find any differences between the groups according to the treatments (vehicle versus the antiviral drugs). This may suggest that there was no prevention of the development of the disease in the pancreas or that there were no functional islets present at the time of transplantation.

The main reason why animals exit the experiment besides the development of ROD was a general reduction in the health status, according to the Hampshire scoring board. Possibly the frequent gavage-feeding may cause tissue damage in the epithelium of the esophagus resulting in loss of appetite and weight loss. However, some animals also outlived the lifetime limit set by the Ethical Committee and was therefore euthanized. It is possible that transplanted animals, not developing ROD stay normoglycemic due to regained function of the islet mass in the pancreas. The proinflammatory process introducing the autoimmune islet destruction may reduce the insulin production without destroying islet cells. Introduction of an islet graft may reactivate the dormant pancreatic islet mass and thus result in prolonged period of normoglycemia. We compared the insulitis scoring to the ROD and found no differences between the groups suggesting that animals not developing ROD do not have a reserve islet mass in their pancreas. We may also assume that no significant remaining islet mass in the pancreas could result in delay of recurrence of disease. This indicates that it is the islet graft supplying the insulin needed to maintain normoglycemia.

No experimental groups had intact islet mass in the pancreas upon euthanization and therefore we can assume that there was no pancreatic islet mass to start with upon the time of the transplantations. No animals in this study had intact pancreatic islet mass or an islet mass in
early inflammatory state upon the endpoint of the experiment. Also; animals euthanized without developing recurrence of disease revealed nothing but a pancreatic islet mass in a late stage of inflammatory destruction.

When we compared the vehicle and antiviral treatment to the outcome of ROD there was no significant difference. This means there is no long term protective or curing effect of the treatment. But even if the treatment with this antiviral substance will not be a cure for the disease we wanted to look at the correlation with the treatment and survival over time. Here a statistical difference was seen in those animals developing ROD, indicating that there is an effect on the progression of disease contributing to delay the ROD.

**Conclusion**

- Diabetic animals selected to enter the study has acquired autoimmune diabetes with destruction of the islet cells. Also, the inflammatory process in the islets is severe with no intact islet mass.

- Also, in animals not developing ROD, the inflammatory process in the islet is severe and proceeding at a late stage in islet cell destruction, thus the prevention of disease development seen in some transplanted mice is not a result of remaining pancreatic islet mass.

- The antiviral drug treatment does not prevent the development of recurrence of disease but may affect the disease development, resulting in a prolonged survival of the transplanted animals.

If this experiment will be applicable to human disease remains to be seen. An animal model will always just be a model and its relevance to the human pathophysiology must always be considered. The individual differences in the immune response among humans and the enormous genetic variation in between viruses may contribute to the large diversity seen in these types of studies. Further research will be needed to examine whether we can be able to anticipate and prevent possible pathological virus strains in type 1 diabetes.
References


