Prevention of Type 1 Diabetes Mellitus in Experimental Studies

BY

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ABSTRACT


The aim of the study was to examine the immune response and different immunoprotective strategies in experimental type 1 diabetes mellitus. The autoimmune destruction of the insulin-producing pancreatic β-cells that leads to type 1 diabetes is complex and incompletely understood. Activated immune cells infiltrate the pancreatic islets at an early stage of the disease and they produce and release cytokines, which may contribute to β-cell dysfunction and death.

Several immunomodulatory agents with different mechanisms have recently been developed in order to suppress cytokine function such as MDL 201, 449A, a novel transcriptional inhibitor of TNF-α. At least in rodent β-cells, many of the toxic actions of cytokines depend on the synthesis of nitric oxide (NO). Aminoguanidine (AG), an inhibitor of NO formation, might therefore be an interesting compound for prevention of type 1 diabetes. Another substance that could influence the course of events leading to this disease is the pituitary hormone prolactin (PRL), since it has the ability to activate different immune cells. We have studied the effects of AG, PRL and MDL 201, 449A on the development of hyperglycaemia and pancreatic insulitis in multiple low dose streptozotocin induced autoimmune diabetes in mice. The natural course after syngeneic islet transplantation of pancreatic islets in NOD mice, a model of type 1 diabetes mellitus was also investigated. AG and PRL were also studied in vitro on cultured isolated rodent pancreatic islets.

We suggest that the insulin-producing cells are specifically targeted by the inflammatory response after syngeneic islet transplantation in type 1 diabetic mice. Our data do not exclude a role for NO in type 1 diabetes, but it raises concerns about the use of AG as a therapeutic agent since an increased mortality and no decline in diabetes frequency was observed. AG did not seem to be directly harmful to β-cell function, but it could affect pancreatic and islet blood flows. PRL and MDL 201, 449A could both counteract hyperglycaemia and insulitis in the early phase of autoimmune diabetes.

Key words: Cytokines, inducible nitric oxide synthase, nitric oxide, NOD mice, pancreatic islets, prolactin, syngeneic islet transplantation, tumour necrosis factor alpha, type 1 diabetes.

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“Science never gives up searching for truth, since it never claims to have achieved it.”

Terry Delovitch, Canada, speech at the 5th International Congress of the Immunology of Diabetes Society in Chennai, India 2001
This thesis is based on the following papers, referred to in the text by their Roman numerals:


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ABBREVIATIONS

AG  aminoguanidine
APC  antigen presenting cell
BB rat  BioBreeding rat
BC  bromocriptine
BSA  bovine serum albumin
cDNA  complementary deoxyribonucleic acid
FCS  foetal calf serum
Hsp 60  heat-shock protein 60
ICC  islet-like cell cluster
IFN-γ  interferon gamma
IL-1β  interleukin-1 beta
L-NAME  Nω-nitro-L-arginine methyl ester
L-NMMA  Nω-monomethyl-L-arginine
MHC  major histocompatibility complex
mRNA  messenger ribonucleic acid
NO  nitric oxide
NOD  non-obese diabetic (mouse strain)
NOS  nitric oxide synthase;
c, constitutive
e, endothelial
n, neuronal
i, inducible
PARP  poly (ADP-ribose) polymerase
PCR  polymerase chain reaction
PRL  prolactin
RIA  radio immuno assay
STZ  streptozotocin
TCR  t-cell receptor
Th  t-helper
TNF-α  tumour necrosis factor alpha
1 INTRODUCTION

1.1 Type 1 Diabetes Mellitus

Type 1 diabetes is the classical insulin-dependent form of diabetes. Thus, these patients cannot survive without insulin treatment. Most patients with type 1 diabetes present young, and the peak of onset is early in puberty. The first acute symptoms are thirst, polyuria, tiredness and weight-loss. The prevalence shows a marked, geographical variation, ranging about 30-fold from the highest frequencies in the Nordic countries to the lowest frequencies in Asia (1-3). In Sweden, the mean incidence among children and young adults is 20-30 per 100 000 and type 1 diabetes in the whole population accounts for about 10-15% of total diabetic cases e.g. 20 000-30 000 individuals (4-7).

Type 1 diabetes develops due to a complex interaction between environmental factors and a genetic predisposition to the disease. Studies in monozygotic twins suggest that the genetic component accounts for 30-40% of the total risk (8-10). Possible environmental triggers are viral infections (e.g. rotavirus (11) and different enteroviruses such as Coxsackie B (12)), dietary factors (e.g. neonatal exposure to BSA in cow’s milk and chemical toxins in food) and stress (13, 14).

40-50% of patients with long-standing type 1 diabetes will develop significant vascular complications such as retinopathy, nephropathy, neuropathy and atherosclerosis, depending on the duration and severity of hyperglycaemia and individual susceptibility (6,15). The main causes of premature death are renal failure and coronary heart disease.
1.2 Pancreatic $\beta$-cell destruction

1.2.1 Autoimmunity

Type 1 diabetes is considered in most cases to result from an autoimmune destruction of the insulin-producing pancreatic $\beta$-cells, leading to an absolute deficiency in insulin synthesis and secretion (16). The autoimmune $\beta$-cell destruction may persist over a prolonged period prior to diagnosis of the disease, but loss of $\beta$-cell mass often accelerates markedly about 6 months before clinical presentation (17, 18). The autoimmune response can be anticipated to develop for several reasons that essentially could be divided into five categories (19):

1) **An undetected infection** where the immune response is directed entirely against an undetected pathogen, but it destroys the infected organ. For example hepatitis C and D are associated with autoimmunity (20). It has also been reported that many patients with psoriasis improve when treated with tetracycline or other antibiotics (21).

2) **Molecular mimicry** may be involved because certain antigens on the surface of the $\beta$-cell can be recognised by antibodies against exogenous antigens. In line with this, antibodies against Coxsackie B virus proteins may crossreact with specific peptide sequences in $\beta$-cell proteins such as GAD.

3) **Uncontrolled cell death**, caused by non-pathogenic toxic agents or mutations in any of the many genes controlling normal programmed cell death (apoptosis) or the scavenging process, could lead to immune cell activation. Thus, the autoimmune response occurs because of cellular distress.

4) **A defective regulation of the immune response** could shift the normal pattern of released antibodies and cytokines in the tissue. The $\beta$-cells are more sensitive to some cytokines and subsequently generated free radicals, than other cell types, and may therefore be damaged.

5) For some autoimmune diseases the **immune response seen may be a consequence, rather than a cause, of cell damage**. Thus, although autoantibodies may be detected, they play no role in the pathogenesis of the disease. In that case, the autoantibodies can serve as diagnostic markers and/or predict disease outcome, but the autoantibodies do not cause the disease or damage.

Circulating autoantibodies against antigens such as GAD, insulin and uncharacterised cell-surface components are detectable in most newly diagnosed patients (17, 22). These autoantibodies might have been present for many years and they are also found in some subjects with apparently typical non-insulin dependent type 2 diabetes mellitus (23).
1.2.2 Cytokines

Infiltrating immune cells produce and release inflammatory mediators e.g. cytokines which may contribute to β-cell dysfunction and death (24, 25). Cytokines are small polypeptides. Like hormones, they act as messengers between cells in order to control the immune system, inflammation, cell growth and haematopoiesis. Most cytokines act locally, but some e.g. IL-1β, IL-6 and TNF-α may also have systemic effects. A cytokine is often produced not only in immune cells but also in other cell types such as stromal cells and epithelial cells. Paradoxically, part of the β-cell destruction might be caused by cytokines produced by the β-cells themselves (26).

Several cytokines are expressed in animal models of type 1 diabetes (24, 27, 28) and the pattern of the network in which these cytokines co-operate is very complex. A specific cytokine might either amplify or counteract the effects of other cytokines. Moreover, the action of a cytokine can be concentration and time dependent.

1.2.3 Th1 & Th2

The autoimmune response in type 1 diabetes includes both cell-mediated (Th1) and humoral (Th2) elements, and the balance between these two pathways has been suggested to determine the outcome of the inflammatory process (18, 29, 30). Activated macrophages and T-lymphocytes of the Th1 subtype produce and secrete cytokines such as IL-1β, IFN-γ and TNF-α, whereas Th2 cells secrete cytokines like IL-4, -5, -6, -10 and 13 (31, 32). This is schematically outlined in Fig. 1.

In both spontaneously diabetic NOD mice and BB rats, an increased expression of Th1 cytokines appears to correlate with β-cell destruction and diabetes development (33-38). On the other hand, the expression of Th2 cytokines correlates with a benign, non-destructive insulitis characterised by an immune cell infiltration around the islets without significant loss of β-cells (33, 34).

Very little is known about the cytokine pattern in the vicinity of human pancreatic islets during the development of type 1 diabetes, but several studies have been performed in vitro in order to study human islet response to cytokines, which has given somewhat different results as compared to rodent islets. Thus, while IL-1β has an early stimulatory (1-2 h) and late (12-24 h) inhibitory effect on rat islets (39), the stimulatory effect is maintained for at least 7 days in human islets, with an increase in both basal and glucose induced insulin release in the presence of the cytokine (40). As in rodent islets, few inhibitory effects are observed when human islets are exposed to TNF-α and IFN-γ alone, but when these cytokines are added in combination with IL-1β, both functional suppression (41-43) and cell death by apoptosis is observed (44).
1.2.4 Costimulation

At least two signals are required in order to activate a resting immune cell (19, 45, 46);

**Signal 1)** Binding between TCR and MHC + antigen

**Signal 2)** Costimulatory signals

The affinity of an individual TCR for its specific MHC-antigen peptide complex is
believed to be relatively low and interaction of complementary pairs of accessory
molecules in the plasma membrane of these immune cells such as B7-CD28 and CD40-
CD154 is needed in order to stimulate activation and proliferation further (45). After this
activation, no further costimulation is needed and these T-cells proliferate in response to a
single signal (45).

If no initial costimulation takes place the T-cell will become anergic, i.e. unresponsive to
any further stimulation by antigen.

The principle that two signals activate but one may induce anergy in an antigen-specific
cell provides potential for targeted immunosuppressive therapy. Thus, activation of resting
T-cells can be blocked by anti-B7 (47) or anti CD-154 (48).

The APCs can also be activated by unspecific endogenous alarm signals from distressed or
damaged bodily tissues (19). These alarm signals may come in pre-packed or inducible
form. The former might be any structure that is normally found inside but not outside the
cell such as DNA, RNA, mitochondria etc. Inducible alarm signals may include heat-shock
proteins and some cytokines such as IL-1 and IFNγ.

1.2.5 Nitric Oxide

NO is the smallest known bioactive product of mammalian cells and can be produced by
most cell types. It is an uncharged gas molecule, which has a high diffusion capacity and
readily crosses cellular membranes. It has been identified as a cellular messenger and
putative neurotransmitter (49, 50) and this discovery was rewarded with the Nobel Prize
1998. Moreover, NO is a free radical that easily reacts with other molecules, especially
those containing an unpaired electron, e.g. oxygen, iron, sulphur and nitrogen. For some
enzymes, reaction with NO results in activation, whereas in others it inhibits the function
of the protein. NO may also destroy iron-sulphur centres (4Fe/4S) within proteins
important for the function of enzymes involved in the Krebs cycle, the mitochondrial
respiratory chain and DNA synthesis/repair.
Figure 1. Simplified schematic drawing of Th1/Th2 response in type 1 diabetes.
Nitric oxide synthase (NOS) catalyses the oxidation of arginine to yield citrulline and NO (51, 52) (Fig. 2). NOS exists in at least three different isoforms; two are mostly constitutively expressed (cNOS), whereas one is inducible (iNOS (=NOS II)) (53, 54). The constitutive isoforms of NOS mediate endothelium-dependent relaxation (eNOS (=NOS III)) (52) and neural transmission (nNOS (=NOS I)) (55) and they are regulated by intracellular Ca \(^{2+}\) levels. eNOS is probably the most important endothelial derived relaxing factor in the body, and its presence is necessary to maintain a normal tissue blood flow (56). NO is produced in much larger amounts by iNOS, which is Ca \(^{2+}\) independent, and appears to mediate the cytotoxic actions of macrophages on target cells (57).

![Nitric Oxide Synthase](image)

**Fig. 2 Synthesis of nitric oxide from arginine.**

Several of the cytokines induce iNOS production in different cell types, acting alone or in combination (58-61). While addition of IL-1β alone or in combination with TNF-α, induces the expression of the iNOS gene in rodent islets (62), a combination of two (IL-1β + IFN-γ) or three (IL-1β + IFN-γ + TNF-α) cytokines is required for iNOS activation in human pancreatic islets (63). At least for rodent β-cells, experiments *in vitro* support the notion that the harmful action of the cytokine IL-1β requires generation of NO and this effect can be counteracted by inhibitors of NOS (39, 64-67). Furthermore, rodent β-cells seem more vulnerable to NO than other cell types (68). The role of NO in human islet destruction is controversial and contradictory results exist (41, 42, 63).

The role of NO following exposure to cytokines may be more complex than initially anticipated. During certain conditions it has been observed that cytokines may induce inhibitory actions in β-cells even when NO production is blocked (42, 43, 69). However, it has been demonstrated that β-cells of mice lacking inducible nitric oxide synthase (iNOS -/-) were not suppressed by exposure to a high concentration of IL-1β for 24h (70). Moreover, these mice had reduced sensitivity to multiple-low dose STZ induced diabetes (70) and β-cells of iNOS -/- appeared also to be resistant to cytokine induced necrosis, but not apoptosis (71). However, in experiments with isolated pancreatic islets from iNOS -/- mice it was observed that prolonged exposure (48 h) to IL-1β can cause inhibition of insulin secretion, despite that NO was not formed (72). Surprisingly, IFN-γ seemed to counteract this effect of IL-1β.
1.3 Is there a Cure for Type 1 Diabetes Mellitus?

1.3.1 Prevention

Many attempts have been made to control the autoimmune attack on the β-cells (17, 73, 74) and there are several ongoing diabetes prevention trials worldwide. Generally it is preferable to start a specific immunomodulatory treatment while substantial β-cell mass remains i.e. during the prediabetic phase. The vitamin B-complex nicotinamide is currently undergoing a multicentre trial in Europe (ENDIT) (75, 76). Nicotinamide is thought to protect against β-cell damage by acting as an antioxidant and thus inhibits the deleterious effects of free radicals. It also inhibits the enzyme PARP, thereby saving the cellular stores of NAD (Fig. 4) (77). Furthermore, it stimulates islet cell proliferation (78). Another interesting immunosuppressive compound which has shown encouraging results in newly diagnosed patients is cyclosporin A, which acts by inhibiting T-helper lymphocyte function (79-81). Unfortunately cyclosporin A must be given early and it has potentially serious side effects, including a toxic action on the β-cell itself (82). Newer immunosuppressive drugs, such as FK-506, are under investigation, and some of these problems may be avoided (83, 84). Moreover, Bacillus Calmette-Guerin (BCG), a non-specific immunostimulant, has been shown to induce extended remission in newly diagnosed patients (85), by an unknown mechanism. In the American Diabetes Prevention Trial insulin is given at low subcutaneous dosages in order to sensitize the immune system and induce a “protective” Th2 humoral response (86) during the prediabetic phase. Insulin is also given orally to another group of patients in this study. The insulin treatment may also "rest" the β-cell and so reduce the expression of “provocative” β-cell autoantigens (87, 88).

1.3.2 Transplantation

Considerable advances have been made in the technology of transplanting either pancreas or preparations of islet tissue, but major problems remain in obtaining donor tissue and in preventing immune rejection of the graft. Nevertheless, transplantation is as yet the only available treatment that can lead to insulin independence. Recently new promising results have been obtained (89), but human islet allograft transplantation can still not be used on a large scale in clinical practice. The results after whole pancreas transplantation are good, with a 1-year graft survival of 85-90% (90). Islet transplants seem to be much more vulnerable (91, 96). Many of them fail within few weeks or months after engraftment and most islet transplants (>90%) have failed within 1 year (83, 91, 92). The reasons for these functional failures are largely unknown, although insufficient number of islets, engraftment difficulties, chronic rejection and recurrence of autoimmune disease have been suggested to be contributing factors (92-95). Moreover, hyperglycaemia in the recipient after transplantation has been shown to deteriorate islet graft survival and function (96).
One of the major obstacles for clinical islet transplantation is the lack of donors. Therefore, it is important to optimise the number of β-cells harvested from each donor. Another possibility is to stimulate the growth and/or differentiation of β-cells, or to genetically manipulate insulin producing cell lines for transplantation. Several studies have shown that differentiated β-cells still have the ability to proliferate at a low pace (97-100). The proliferation rate can be affected in many ways, for example by growth stimulating hormones e.g. GH and PRL (101-104). Also the size and composition of the graft and the blood glucose level in the recipient are of crucial importance for β-cell replication (105).

1.3.3 Future perspectives

Other fascinating fields of diabetes prevention, which have attracted much attention lately, especially in public media, is gene therapy and diabetes “vaccination”. Gene transfer techniques are now being tested in insulin producing cells aiming to design efficient and safe vectors that could transform β-cells, and thereby protect the remaining β-cell mass in newly diagnosed diabetics or pre-diabetic individuals at high risk (106). Attempts are also being made to genetically manipulate cell lines to become artificial β-cells (107) or to stimulate differentiation from pancreatic stem cells (108). Such cells could compensate for the lost endogenous cells and restore insulin secretion.

Another tentative strategy in order to prevent type 1 diabetes is to stimulate the immunoregulatory mechanisms before onset of the disease, in some cases comparable with vaccination. No data from humans has still been reported, but studies in animal models have revealed that microbial agents, autoantigens and autoantibodies often have a protective action against diabetes development. The protective effects against diabetes may result from activation of a humoral Th2 response and consequently downregulation of the cell-mediated Th1 autoimmune response. Antibodies against β-cell antigens might bind to the surface of the β-cell, and perhaps also bind to the epitope presented in association with antigen presentation and prevent T cell recognition, thereby delaying disease onset (109-111).
The overall aim of this thesis was to explore new strategies in the prevention of type 1 diabetes mellitus. The more specific aims were as follows:

* To evaluate the effects of different immunomodulatory drugs on isolated pancreatic islets and on the development of diabetes in animal models of type 1 diabetes.

* To evaluate the acute effect of the NOS inhibitor aminoguanidine (AG) on pancreatic and islet blood flow in anaesthetised rats, since this could have a role in the mechanisms leading to β-cell destruction.

* To study the immune response after syngeneic islet transplantation in NOD mice.
3 MATERIALS & METHODS

3.1 Animal Models of Type 1 Diabetes Mellitus

The Animal Ethics Committee in Uppsala had given its approval to all animal experiments in this thesis.

3.1.1 Streptozotocin

Streptozotocin (STZ) is a commonly used substance in animal models for the study of diabetes, but originally it was used as an antibiotic. STZ was isolated from *Streptomyces achromogenes* (112) in the early 1960’s and it was found to be an effective broad-spectrum antibiotic drug (112, 113). It was also discovered that STZ also possessed anti-tumour (114), diabetogenic (115) and oncogenic (116) properties.

In 1976, Like and Rossini reported that multiple low dose injections of STZ in mice produced pancreatic insulitis, with β-cell destruction and diabetes mellitus (117). They suggested that STZ initiated a cell-mediated immune reaction. Particles of type C viruses were also found within the β-cells and this observation evoked the hypothesis that STZ may activate murine leukemia retrovirus. Later, it was observed that macrophages were present in the pancreatic islets very early after multiple STZ injections in all mice developing hyperglycaemia (118). One injection of a high dose STZ induces β-cell necrosis within 4 h of administration and hyperglycaemia is achieved rapidly, whereas in contrast, multiple low dose injections of STZ induce a gradual elevation of blood glucose in certain mouse strains (117). This animal model appears to depend on both direct β-cell toxicity of STZ and an autoimmune component.

![Fig. 3 Structure of streptozotocin](image)

The STZ molecule consists of a glucose residue with a methylated nitrosourea linked to carbon-2 (Fig 3). It has a short biological half-life *in vivo* of 5-15 min (119, 120). It is possible that the glucose entity of the molecule facilitates transport over the plasma membrane into the cytosol of the β-cell via glucose transporter proteins.
Inside the cell, STZ is decomposed. During this procedure, reactive methylcarbonium ions are produced, which may alkylate the DNA and cause cross-links between the DNA strands (121) (Fig. 4). This initiates DNA repair mechanisms in the cell. The damaged sections of the DNA are recognised and excised by DNA repair nucleases, leading to DNA strand interruptions (122).

It has been suggested that a nuclear enzyme called poly(ADP-ribose)polymerase (PARP) participates in this process (123). PARP and DNA polymerases compete for binding to the strand breaks in the DNA (Fig. 4), but PARP binds stronger which affects the access for repair enzymes to the damaged site. It is thought that binding of PARP prevents replication and transcription of damaged DNA (124) and it may also constitute an “emergency signal”.

If PARP binds, it leads to poly(ADP-ribose) synthesis and long chains of NAD become attached to PARP, and maybe also to other acceptor proteins, and thereby these proteins are modified (123). This kind of modulation by ribosylation is a way of changing binding properties of proteins (125, 126). The automodified PARP has reduced affinity for the DNA and therefore it dissociates, allowing the DNA repair enzymes to reach the damaged area (124). DNA polymerase then synthesises a new strand, and the DNA parts are finally joined together by DNA ligase (122).
The DNA damage, caused by STZ, and the following activation of DNA repair mechanisms sooner or later depletes the cell content of NAD (127-131), not only in \( \beta \)-cells, but also in liver cells (119). This causes a deficiency in cofactors for oxidative phosphorylation with subsequent lack of ATP, which in turn causes diminished protein synthesis, insulin release and reduced activity of the ion pumps, which can lead to cell death. In this context, it has been shown that the action of PARP can be inhibited by nicotinamide and theophylline, thereby saving the cellular NAD stores but at the same time deteriorating the DNA repair (77). Furthermore, it has recently been reported that mice lacking the PARP gene are resistant to pancreatic \( \beta \)-cell destruction and diabetes development induced by STZ (132).

**Multiple low dose STZ treatment *in vivo***

The mice received first either an injection of saline or STZ (40 mg/kg body weight). (Fig. 5). After 30 min, the mice were given a second injection of saline or intervention drug. The first injection was given daily for 5 days, whilst the second injection was given daily for different periods of time as specified in papers I, III and IV. Blood glucose was subsequently monitored during the experimental period.

![Multiple low dose STZ diabetes model in mice](image)

*Figure 5. Multiple low dose STZ diabetes model in mice*

(Modified illustration from Urs Christen, The Scripps Research Institute, La Jolla, CA)

**STZ induced islet dysfunction *in vitro***

In paper III cultured pancreatic islets were incubated at 5.6 mM glucose and different concentrations of intervention drug and then exposed to STZ for 30 min. A low glucose concentration (5.6-11 mM) is preferable because a high concentration of glucose (>11 mM) may diminish the toxic effect of STZ. After overnight culture, glucose-stimulated insulin release of the islets was examined.
3.1.2 Non-Obese Diabetic Mice (NOD)

In 1974, a Japanese research group were trying to establish a spontaneous eye cataract strain in mice (Shionogi Research Laboratory in Osaka). Since cataract is a common complication to diabetes, they started to screen for hyperglycaemia and glycosuria. They found a female mouse with these symptoms and started to back-cross with siblings. The end result of this selection was an inbred model of pre type 2 diabetes. The non-obese diabetic (NOD) strain was subsequently derived from a co-selected control line, exhibiting normal fasting blood glucose levels. The first case of spontaneous autoimmune type 1 diabetes occurred unexpectedly at the 20th generation of sibling mating. It may be interesting to note that at the same time its “Bigger Brother” the BioBreeding (BB) rat was also established as a model of type 1 diabetes.

In 1980 Makino et al described the new NOD strain of mice that became spontaneously diabetic in a manner that clinically resembled the disease in humans (133). This was clearly an advantage compared with earlier models such as the multiple low doses STZ model. Since diabetes occurred spontaneously, it was easier to rule out non-specific toxic components in the disease process, as well as studying correlation to genotype. After some struggle, the first NOD mice were brought to Uppsala Biomedical Centre in 1988 from Clea Company, Aobadi, Japan, under protection of my supervisor, Stellan Sandler. Later on the NOD mouse has become extensively used worldwide. In fact, the NOD mouse model is now by many being considered “as good as it gets” in type 1 diabetes research (134).

The NOD mouse genome is well defined and has striking similarities with human diabetes susceptibility genes (135-138). It has also been found that it has less geno- and phenotypic variation than other animal models e.g. BB rat (134). However, the genetic homogeneity can not reflect the genetic diversity in the human disease and might even be considered as “a single case study”.

The pancreatic insulitis prior to and during onset of type 1 diabetes has been observed both in humans and NOD mice, although histological studies of human subjects are very limited. The immune cell infiltrates in NOD mice have a denser pattern and contain a larger proportion of leukocytes compared to humans (134, 139). The first lesions of insulitis in NOD mice usually occur at 3-4 weeks age and hyperglycaemia can be detected at 8-12 weeks of age in the typical case. Some animals get insulitis without developing hyperglycaemia.
There is a more pronounced gender bias in disease manifestation, compared to both humans and BB rats. Approximately 80% of female and 10% of male NOD mice become diabetic under pathogen-free conditions, but castration of males at an early age has been reported to increase the incidence of diabetes in male NOD mice (140). Furthermore, it has been reported that from the onset of insulitis until the development of overt diabetes, IFN-γ mRNA in the islets from females were significantly higher than in males. In contrast, IL-4 mRNA levels were lower in females than in males (33). These findings support the hypothesis that Th1 cytokines correlate with β-cell destruction and diabetes development, whereas Th2 cytokines correlates with a non-destructive insulitis (33, 35-38).

Macrophages and dendritic cells are the first cell types infiltrating the islets in NOD mice (141) as well as in the BB rat (142). The presentation of β-cell-specific autoantigens in association with MHC class II molecules by macrophages to CD4+ T-helper cells, is considered to be the initial step of the cell-mediated Th1 response leading to the development of the disease (Fig. 1)(143). In addition, the activated macrophages secrete cytokines such as IL-1, IL-6 and IL-12, which are necessary for the activation of CD4+ T-helper cells. The stimulated CD4+ T-helper cells secrete IFN-γ and IL-2. IFN-γ activates other macrophages, which in turn release cytokines such as IL-1β, TNF-α and free radicals. IL-2 from stimulated CD4+ cells induces proliferation and migration of peripheral T-cells to the inflamed area. Precytotoxic CD8+ T-cells with β-cell specific autoantigen receptors differentiate into cytotoxic T-cells upon recognition of a β-cell specific peptide plus MHC class I on the β-cells, in the presence of IL-1 and IL-6. The cytotoxic CD8+ T-cells may then damage the β-cells by releasing perforin and granzyme, and by Fas-mediated apoptosis (143, 144). In this way macrophages, CD4+ T-cells and CD8+ T-cells synergistically destroy β-cells, resulting in the onset of autoimmune diabetes.

During the humoral Th2 response, which also might be involved in the inflammatory process, antibodies on the surface of B-lymphocytes will bind β-cell specific autoantigens (Fig. 1). IL-4, IL-5, IL-6 and IL-10 secreted from T-helper cells activate B-lymphocytes presenting a combination of MHC class II and the processed antigen on their surface, which the T-helper cell receptor will bind to. This event leads to activation of the B-lymphocyte and it will start to proliferate and produce antibodies on a large scale (28, 145).
3.1.3 Recurrence of Disease

Transplantation of islets has been extensively investigated in the NOD mouse. In paper V, a NOD mouse model designated "recurrence of disease" was studied. This model is a way of controlling the time of onset for the development of hyperglycaemia and insulitis in NOD mice.

In this model, about 500 syngeneic islets from young nondiabetic NOD mice are transplanted under the kidney capsule in a diabetic NOD mouse. A few days after transplantation, the blood glucose levels are normalised, but raise again after 1-2 weeks. The islets become inflamed after 4-6 days (146, 147). This rejection of the grafts may depend on recurrence of disease (93, 95, 148), which means that the original diabetic disease returns, because memory cells in the immune system recognise and induce destruction of the transplanted islet cells. The inflammation is considered to be specific because syngeneic pituitaries do not get inflamed, when they are transplanted in the same manner (148).

The recurrent immune reaction is particularly strong and can only partially be suppressed by downregulation of Th1 cytokines (149) or blockade of costimulatory pathways of T-cell activation (48). It is also relatively resistant to cyclosporine A (150). Recurrence of disease is not only seen in NOD mice, but also in human pancreas transplantation (94) and in the BB rat model of diabetes (95) and it has several applications in studies of insulitis development and attempts of therapeutic intervention.
3.2 Islet studies

3.2.1 Isolation and culture of rodent islets

Pancreases from rats and mice were digested with collagenase and islets picked by hand (paper I, II, III and V). Groups of 150-200 islets were precultured for 4-7 days before the experiments were performed. The medium was changed every second day.

Previous studies suggest that glucose may modify a putative stressful action on islet $\beta$-cells in vitro (151-153), and different opinions exists about the role of NO in insulin secretion (154-157). In paper II experiments on islets cultured both at 11.1 mM glucose and 28 mM glucose in the presence or absence of aminoguanidine (AG) were performed. However, AG was not tested at lower glucose concentrations because it is well established that culture in medium at lower glucose concentrations (e.g. 5.6 mM) impairs rodent $\beta$-cell function (151, 158).

3.2.2 Islet glucose-stimulated insulin release and glucose oxidation

For the insulin release experiments, islets were incubated at 1.7 mM glucose during the first hour followed by 16.7 mM glucose during the second hour. The islets were then retrieved for determination of insulin and DNA content.

In order to measure the glucose oxidation, islets were incubated in medium supplemented with radioactive and non-radioactive glucose to a final concentration of 16.7 mM glucose (159). After 90 minutes, the islet metabolism was arrested by the addition of antimycin A. Radioactive CO$_2$ formed by cell metabolism was released from the incubation medium by injection of NaHPO$_4$ and trapped in hyamine hydroxide during a further 120 min incubation. Finally, scintillation fluid was added and the radioactivity measured.

3.2.3 Measurement of insulin and DNA

In order to measure the content of insulin and/or DNA, groups of 30 islets were homogenised in water with ultrasound. A fraction of each lysate was mixed with acid ethanol and the insulin was extracted overnight (4°C). The insulin concentration of the extract was measured by RIA (160). RIA was also used to measure insulin released into the culture medium. The intra- and interassay coefficients of variation in the RIA are 14% and 9% respectively. DNA in the cells was determined by a fluorometric method described by Hinegardner (161).
3.3 Blood Flow Measurements

In paper II measurements of blood flows were performed by a microsphere technique, in combination with a freeze-thawing technique that enables visualisation of the pancreatic islets and microspheres (162, 163). These nonradioactive microspheres have a diameter of 11 µm, which is somewhat larger than an erythrocyte (7 µm), causing the microspheres to fasten in the capillary networks of the body. The number of microspheres present in an organ will therefore be proportional to the blood flow in that area. Briefly, the microspheres are injected into the circulation via the ascending aorta. Meanwhile, an arterial reference sample is collected from a catheter in the femoral artery by free flow (≈ 0.5 ml/min). The adrenal glands and the pancreas are removed, weighed and treated with a freeze-thawing technique (162) that allows a general visualisation of the tissue morphology and counting of microspheres. The number of microspheres in the arterial reference sample is determined by transferring the blood to glass microfibre filters and counting the microspheres in a microscope equipped with transmitted light. The blood flow values are then calculated by comparing number of microspheres present in the organ and number of microspheres in the reference sample. The microsphere content in the adrenal glands is counted to confirm that the microspheres were adequately mixed in the circulation. A difference of <10% in blood flow values between the glands was taken to indicate sufficient mixing.

3.4 Histology

3.4.1 Routine Histology Staining and Morphologic Examination

Pancreatic glands and/or transplants were removed and fixed in formalin and embedded in paraffin. Sections were cut and stained with hematoxylin and eosin and/or immunohistochemistry for islet hormones (Paper III, IV, V and I). The lymphocyte infiltration and hormone content was ranked according to arbitrary classes as previously described and illustrated (164) in Paper III, IV, V and I.

3.4.2 Immunohistochemistry of frozen sections of transplanted islets

In paper V, islet grafts were excised and frozen at −70°C at different time points after transplantation. Serial sections were cut, air dried and then stored at −70°C. After storage, the slides were fixed in acetone. The stainings were performed using a staining robot, DAKO Tech Mate 500 Plus. Unspecific antibody binding was blocked by incubation with normal rabbit serum. Subsequent incubations of the sections were carried out: incubations with monoclonal rat antibodies for CD4, CD8 and F4/80, were followed by rabbit anti-rat IgG antibody. After a final incubation with monoclonal rat alkaline phosphatase-anti-alkaline phosphatase reagent, the alkaline phosphatase reaction was developed. Omitting the primary antibody served as control experiments. The extent of immune cell infiltration and amount of insulin positive cells was evaluated as described in paper V.
3.5 RT-PCR

Spleen cells (paper IV) and islet grafts (ongoing study) have been used for reverse transcription polymerase chain reaction (RT-PCR) examinations of cytokine mRNA encoding IL-4, IL-10, IFN-γ, TNF-α and β-actin (control).

RNA was isolated using RNeasy Mini Kit (Qiagen). Briefly, the samples were first disrupted in the presence of a highly denaturing guanidium isothiocyanate (GITC)-containing lysis buffer that immediately inactivates RNases to ensure isolation of intact RNA. Ethanol was added to provide appropriate binding conditions and the lysate was then applied to a spin column where total RNA binds to a silica-gel-based membrane and contaminants were washed away with specialised high-salt buffers. RNA was then eluted in water and reverse transcribed into cDNA using the standard protocol for SDS Reverse Transcription System with oligo(dT) primers.

We recently started to use a new rapid method for RNA and cDNA isolation in which oligo(dT)-coated manifold support allows mRNA to be isolated directly from cell lysates by hybridisation followed by transfer through different washing and enzymatic steps of the assay with minimal pipetting. (165, 166). Professor Ulf Landegren and colleagues at the Department of Genetics and Pathology, Rudbeck Laboratory, Uppsala University have recently developed this method, which greatly simplifies mRNA isolation.

PCR reactions were performed in the ABI Prism 7700 (TaqMan assay*, PE Biosystem). This is one of many new methods to monitor DNA amplification reactions in real time, which has made mRNA quantification by RT-PCR easier and more accurate. The assay takes advantage of the 5'-nuclease activity of Taq DNA polymerase to cleave a dual-labelled probe, which hybridises to the amplified fragment during the extension phase (167, 168) (illustrated below). The cleavage reaction separates a fluorophore (i.e. reporter) from the probe, producing increased fluorescence in the sample. The increase in fluorescence is proportional to the target accumulation and can be measured in real time (169). The amplification cycle at which fluorescence exceeds baseline is called the threshold cycle (C_T). The C_T value is a measure of the number of target molecules in the amplification reaction. The C_T values for each cytokine are subtracted from the C_T values for the housekeeping gene (β-actin) in the sample and these values are used to calculate the relative quantity, compared with controls.

* TaqMan assay

![Amplification plot]
3.6 Interventions

3.6.1 Aminoguanidine

Aminoguanidine, a selective inhibitor of iNOS

Aminoguanidine (AG) and some other guanidines such as N\textsuperscript{\text{\textomega}-monomethyl-L-arginine (L-NMMA) and N\textsuperscript{\text{\textomega}-nitro-L-arginine methylester (NAME), have the ability to inhibit NO formation (Fig. 2), because they are structurally similar to arginine (170, 171) (Fig. 6). AG inactivates all isoforms of NOS, but is considered to be a more potent and selective inhibitor for iNOS (171-175). Concerning its role in prevention of diabetes, AG might interfere with NO generation both in processes leading to \(\beta\)-cell destruction and regulation of blood flow (171). Moreover, AG could have beneficial effects on diabetes-induced vascular changes because it is also able to diminish glycosylation of proteins (176) and it might also have antioxidant effects (177).

\[
\begin{align*}
\text{L-Arginine} & \quad & \text{Aminoguanidine} \\
\begin{array}{c}
\text{NH} \\
\text{C} \\
\text{H}_2\text{N}
\end{array} & \quad & \begin{array}{c}
\text{NH} \\
\text{C} \\
\text{H}_2\text{N}
\end{array} \\
\begin{array}{c}
\text{NH-CH}_2\text{CH}_2\text{CH}_2\text{COOH} \\
\text{H}_2\text{N}
\end{array} & \quad & \begin{array}{c}
\text{NH-NH}_2
\end{array}
\end{align*}
\]

*Fig. 6 Structure of arginine and aminoguanidine.*

The AG molecule is a very reactive compound, which has the potential to interact with several different kinds of carbonyl groups in the organism. The mechanisms by which AG exerts its effects have not been fully elucidated and probably involve more than one mechanism.
Effects of AG on β-cell function

The role of NO in regulation of islet β-cell insulin secretion has been debated (154-157). It has been demonstrated that both cNOS and iNOS are present in rodent islets (178), but NO formation is presumably not particularly important for glucose-regulated insulin secretion from β-cells. NO production in cultured rodent islets in the absence of exogenously added cytokines is very low (64, 65). A decrease in islet NO production induced by AG addition to unstimulated islets must therefore be very small. It has been shown that prolonged exposure to AG in vitro, at low concentrations known to inhibit iNOS (≤ 0.5 mM), is not harmful to β-cells (172, 174). However, higher concentrations of AG (≥ 4.55 mM), that also inhibit cNOS (≥ 0.5 mM), have been reported to impair β-cell function in vitro (42, 179). It is improbable that this inhibitory action mainly is due to NOS inhibition. It has instead, been suggested that AG might cause a lowering of intracellular pH (180), which is likely to decrease insulin release from islets (181, 182).

The ability of AG to prevent or delay the development of type 1 diabetes

It has been suggested that therapeutic interventions, which reduce NO formation, might convey a protective effect against the development of type 1 diabetes. AG has therefore been thoroughly studied by several groups. It is also under evaluation in humans as a prophylactic for some chronic diabetic complications.

The possible beneficial action of AG would be to prevent NO formation from both macrophages and β-cells. It has been shown that AG could prevent IL-1 induced NO formation and inhibitory actions on insulin-producing cells in vitro (171). Furthermore, it has also been reported that treatment of mice with NOS inhibitors e.g. L-NMMA (183) and L-NAME (184, 185) suppressed diabetes in mice treated with multiple low doses of STZ. However, when AG was administered to diabetes prone non-obese diabetic (NOD) mice (66, 186) or BioBreeding rats (BB) (187), it failed to decrease the incidence of diabetes. These observations, among others, raise concerns about its potential use as a drug for clinical use. In this context, it may also be of importance that AG was shown to generate hydrogen peroxide via an inhibition of catalase in studies performed on rat liver cells and human erythrocytes in vitro (188).
3.6.2 Prolactin

Prolactin (PRL), a neuroendocrine peptide hormone (24 kDa) normally associated with lactation, is produced by the pituitary gland and has been suggested to play an important role in the regulation of the immune system because it stimulates lymphocyte proliferation and macrophage functions (189, 190).

PRL receptors have been identified on the cell membranes of white blood cells, and lymphocytes have been shown to secrete a PRL-like substance (190-192). The stimulatory effect of PRL on immunity may result from antagonism of immunosuppressive glucocorticoid effects (193). A severe inflammation often results in elevated levels of circulating glucocorticoids and ACTH (adrenocorticotropin), which in turn, reduces the secretion of PRL (194, 195). PRL promotes the antibody response and increases the production of IFN-γ and inhibits IL-1 production (193). Administration of PRL or bromocriptine (BC) (a dopaminergic agonist decreasing the secretion of PRL (196)) could therefore influence the progression of the inflammatory response.

Several studies have suggested that PRL may be important in the pathogenesis of various autoimmune diseases (191, 197-200), but few have focused on the role of PRL in type 1 diabetes mellitus. In the human, some protective effects of BC on β-cell function were observed in diabetic patients (201). With regard to the experimental animal models of type 1 diabetes, divergent results exist. A protective effect of BC against hyperglycaemia was observed in female NOD mice (202), but, in contrast, the drug appeared to accelerate the onset of diabetes in NOD males and significantly increased the rate of insulitis in both sexes (203). BC also possesses various metabolic effects, for example it produced a marked hyperglycaemia in rodents (203). It also inhibited insulin release from isolated mouse pancreatic islets in vitro, suggesting that insulin release may be controlled by D2-dopaminergic receptors on the β-cells (204). Previous data has shown that PRL stimulates insulin secretion and proliferation of β-cells in islets of murine and human origin (101, 205-207). A possible protective action of PRL in type 1 diabetes could therefore be attributed to PRL-induced stimulation of β-cell.
3.6.3 MDL 201, 449A

TNF-α is a macrophage-derived proinflammatory cytokine (208, 209) (Fig. 7). During the cell-mediated immune response, it acts as an autocrine factor for induction of other proinflammatory mediators, such as IL-1β and IL-6, which enhance macrophage function (210, 211). Substances that specifically inhibit TNF-α may therefore have a therapeutic potential in inflammatory diseases.

![Figure 7. TNF-α – Proinflammatory cytokine](Illustration from Urs Christen, The Scripps Research Institute, La Jolla, CA)

In experimental type 1 diabetes, administration of TNF-α to newborn NOD mice leads to an earlier onset of diabetes but treatment with anti-TNF-α mouse antibody completely prevented the disease (212). In addition, local expression of TNF-α in neonatal NOD mice has been shown to promote diabetes by enhancing islet antigen presentation (213).

On the other hand, there are studies concerning TNF-α and prevention of type 1 diabetes with opposite results. Thus, long-term administration of TNF-α to adult NOD mice and BB rats has been reported to delay the onset and decrease the incidence of autoimmunity (214, 215). Moreover TNF-α has been reported to downregulate type 1 cytokines and prolong survival of islet grafts in NOD mice (216).

A novel transcriptional inhibitor of TNF-α namely MDL 201, 449A, has been shown to be a very effective agent in vitro to selectively inhibit TNF-α secretion from macrophages (217). Moreover, mice susceptible to the development of rheumatoid arthritis-like disease treated with MDL 201, 449A exhibited reduced inflammatory arthritis, autoantibody formation, and serum TNF-α levels after 30 days of treatment (218). In the pig-to-mouse model of xenograft rejection, foetal porcine islet-like cell cluster (ICC) xenograft rejection graft survival was not found to be prolonged, but reduced numbers of CD4+ T-cells were observed in mice treated with MDL 201, 449A (219).

These data suggest that specific transcriptional inhibition of TNF-α with MDL 201, 449A could have a therapeutic potential in treatment of inflammatory diseases.
4 SUMMARY OF RESULTS & DISCUSSION

4.1 Paper I

AG counteracted IL-1β induced inhibition of islet glucose oxidation *in vitro*, but failed to protect against diabetes induced by multiple low doses of STZ.

One possible explanation why AG did not reduce hyperglycaemia or insulitis could be that the dosage of AG was insufficient. Since we observed an increased mortality in the animals given AG in addition to STZ, we did not find it feasible to increase the AG dosage further. The cause of the deaths is unclear. It is possible that AG exerted deleterious circulatory effects and/or that AG interacted in the toxic pathways of STZ. The increased mortality could also be due to a disturbance in the pH homeostasis of the mice (180).

The finding that AG was potent in antagonising the inhibitory actions of IL-1β on islets *in vitro* (220) is probably due to an inhibition of iNOS (176) and/or antioxidant effects (177).

These findings do not rule out that NO might be involved in the process leading to type 1 diabetes, but since we observed an increased mortality in the mice treated with AG + STZ it raises concerns about the potential clinical use of AG.
**4.2 Paper II**

Dose-dependent effects of AG on rat pancreatic islets in culture and on pancreatic islet blood flow of anaesthetised rats.

We were able to show that prolonged *in vitro* exposure (2-6 days) of rat pancreatic islets to AG at low concentrations (0.1-1 mM), that are known to inhibit iNOS (≤0.5 mM), and cNOS (≥0.5 mM), did not affect islet DNA content indicating that AG was not toxic to β-cells. Moreover, the data suggest that insulin production was mainly unaffected at these low concentrations of AG. This may support the notion that NO formation is not a prominent component in glucose regulated insulin secretion from β-cells. However, when islets were exposed to 5 mM AG for 8 days, islet glucose-stimulated insulin secretion was impaired. This indicates that high concentrations of AG, which probably are required to prevent non-enzymatic glycation of proteins related to diabetes complications, inhibit β-cell function. The main *in vitro* findings in paper II are summarised below in Table 1.

<table>
<thead>
<tr>
<th></th>
<th>(mM AG)</th>
<th>Medium insulin</th>
<th>Insulin release</th>
<th>glucose oxidation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>2 days exposure</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 mM glucose</td>
<td>0.1-1</td>
<td>↑ 30-50 %</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td>5-10</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>ND</td>
</tr>
<tr>
<td><strong>6 days exposure</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 mM glucose</td>
<td>0.1-1</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>28 mM glucose</td>
<td>0.1-1</td>
<td>-</td>
<td>-</td>
<td>↓ 15-25 %</td>
</tr>
<tr>
<td><strong>8 days exposure</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>11 mM glucose</td>
<td>5</td>
<td>↓ 50-75%</td>
<td>↓ 50 %</td>
<td>-</td>
</tr>
<tr>
<td>28 mM glucose</td>
<td>5</td>
<td>-</td>
<td>↓ 50 %</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 1. Summary of effects of AG on rat pancreatic islets in vitro**

(- denotes no effect and ND denotes not determined)
It is unlikely that the inhibitory action by AG was attributed to NOS inhibition. It is instead more conceivable that AG caused a lowering of intracellular pH (180) and thereby decreased the insulin release (181, 182).

Pancreatic blood flow was decreased by all doses of AG, but islet blood flow was only decreased after administration of the highest dose, i.e. 50 mg/kg body weight of AG. This is probably due to local regulatory mechanisms acting to maintain a sufficient islet blood flow when the pancreatic blood perfusion is decreased. The administration of a high dose of AG (50 mg/kg body weight) caused a pronounced decrease in both whole pancreatic and islet blood flow, probably reflecting a more complete inhibition of the enzyme. Our findings are compatible with the view that NO formed from both iNOS and cNOS can affect islet blood flow.

When glucose is administered, an increased vagal neural input to the islets occurs, which also mediates the islet blood flow increase (221). It seems as if vagal stimulation of islet blood perfusion can take place, even though only NO formed from cNOS is present within the islets. A basal production of NO within the islet vasculature is probably necessary in order to keep the basal islet blood perfusion markedly higher than that of exocrine pancreas (163). NO produced by cNOS is likely to be needed to maintain the high islet blood flow, whereas under certain conditions, NO formed by iNOS can modulate the islet blood flow (222). If one or both of these enzymes are inhibited, the blood perfusion will decrease, because the effects of vasoconstrictors will be dominating. If this persists over a certain time period, it can have deleterious consequences for both the exocrine and endocrine function of the gland.
4.3 Paper III

Protective effects of PRL on multiple low dose STZ-induced diabetes in vivo, but not on STZ induced islet dysfunction in vitro.

Our results indicate that PRL decreases hyperglycaemia and insulitis in the early phase of autoimmune diabetes. The reason for this effect is unclear, but it could be that PRL in itself enhances β-cell function during a period when the β-cell mass is being reduced, or that PRL counteracts the autoimmune mechanisms leading to β-cell destruction. PRL could not prevent the inhibitory effects of STZ on glucose-stimulated insulin secretion in vitro. It is therefore unlikely that the protective effect of PRL observed in vivo was due to molecular interactions between PRL and STZ, or an interference with the cellular events leading to β-cell damage by STZ.

Previous data has shown that long-term exposure to PRL stimulates insulin secretion and proliferation of β-cells in islets of murine and human origin (102, 205). The possible beneficial action of PRL in the present study could thus depend on PRL-induced stimulation of β-cell function. However, we observed that PRL did not stimulate insulin release in vitro after short-term incubation. Moreover, an accurate quantification of β-cell replication in vivo in an animal with insulitis would be difficult to perform, due to the presence of infiltrating immune cells within the islets.

It is likely that PRL regulated the immune cells in the present experiments. A possible mechanism could be that PRL inhibited IL-1 production from macrophages (193) and thereby promoted an altered immune response by shifting the balance from a cell-mediated to a humoral immune response. This has been proposed earlier to affect the outcome of the autoimmune process in type 1 diabetes (30, 109, 223).
4.4 Paper IV

MDL 201,449A, a novel transcriptional inhibitor of TNF-α, partially prevented hyperglycaemia and reduced pancreatic insulitis in mice injected with multiple low doses of streptozotocin.

However, the compound could not prevent single high dose STZ induced diabetes, which is caused by direct toxicity to the β-cells. In addition, preliminary results from an ongoing study show that MDL 201,449A therapy can not protect syngeneic islet grafts in NOD mice from autoimmune destruction, as has earlier been reported with a IL-1 receptor antagonist (IL-1ra) (224). Whether the effect of MDL 201,449A can be explained on the basis of cytokine ratios is now being investigated.

The experiments with the ConA stimulated spleen cells indicate that the preparation of MDL 201,449A we used had the ability to counteract TNF-α mRNA expression, but it is important to note that the molecular mechanism by which MDL 201,449A inhibits TNF-α production is not fully elucidated.

Moreover, it should be noted that even if TNF-α is of importance in the development of autoimmune diabetes in mice, other cytokines and environmental factors might efficiently substitute for the lack of TNF-α (225). This cytokine is also involved in many other processes in the body. For example, it stimulates tumouricidal activity (208), weight loss (226), insulin resistance (227), has both pro- and anti-angiogeneic activity (228) and it is associated with programmed cell death of oligodendrocytes and other cells (229). Chronic TNF-α blockade might therefore result in several adverse effects such as an increased susceptibility to infections and tumours which raises concerns about therapy with TNF-α inhibition beyond relatively short term and acute interventions.
4.5 Paper V

The number of insulin positive cells in syngeneic islet grafts in NOD mice were substantially decreased over time, while other endocrine islet cells were not significantly affected.

Infiltration of leukocytes and distortion of islet structure was observed 4 days after transplantation, followed by massive immune cell infiltration on days 7 and 10. On day 14 there was clearly visible fibrosis, extensive immune cell infiltration and significant loss of endocrine cells.

F4/80+, CD4+, and CD8+ cells, but no eosinohilic granulocytes were detectable 4 and 6 days after transplantation. These findings support the notion that the early inflammation in recurrence of disease is a specific Th1 mediated response and resembles autoimmunity during the natural course of type1 diabetes in NOD mice (48, 230, 231).

Blood glucose levels varied considerably between individual mice on a given day, but were significantly decreased on day 2 and 4 compared to day 0. The recipients exhibited the highest prevalence of hyperglycaemia at 10 and 14 days post transplantation. On average, at the time of graft removal, hyperglycaemic mice exhibited a lower content of insulin positive cells in the graft than normoglycaemic animals. Furthermore, the expression of CD4, CD8 and F4/80 tended to be higher in mice that were normoglycaemic when the graft was removed, than among those who were hyperglycaemic at that time-point. It can be assumed that the islet grafts in the normoglycaemic animals were more active and secreted more insulin and therefore they expressed more antigen, which was recognized by the memory T-cells in the grafts.
5 GENERAL CONCLUSIONS

I
The present data raises concerns about the use of AG as a therapeutic agent in type 1 diabetes mellitus.

II
AG can affect pancreatic blood flow, but does not seem to be directly harmful to β-cell function at concentrations that inhibit iNOS.

III
PRL may affect hyperglycaemia and insulitis in the early phase of autoimmune diabetes.

IV
Transcriptional inhibition of TNF-α might be an interesting strategy in prevention of type 1 diabetes mellitus.

V
Insulin containing cells are specifically targeted by the inflammatory response after syngeneic islet transplantation in NOD mice, probably due to a recurrence of the autoimmune disease.
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“It is perhaps today, where a certain underevaluation - well, even disrespect - of scientific research is growing - useful again and again to refer to the fact of how well suited the discovery of insulin is to demonstrate that unprejudiced scientific research which may not appear to be of immediate practical value, sooner or later will be fruitful also to clinical practice.”

Oskar Minkowski, speech at the celebration of the Society for Internal Medicine of Rhein-Westfalen, 18 November, 1929