Enterovirus Infections of $\beta$-Cells

A Mechanism of Induction of Type 1 Diabetes?

ANNA-KARIN BERG
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Abstract

The process of β-cell destruction that leads to type 1 diabetes (T1D) is incompletely understood and it is believed to be a result of both genetic and environmental factors. Enterovirus (EV) infections of the β-cells have been proposed to be involved, however, the effects of EV infections on human β-cells have been little investigated. This thesis summarises studies of three different Coxsackie B4 virus strains that have previously been shown to infect human islets. The effects of infections with these EV were studied in vitro in human islets and in a rat insulin-producing cell line. In addition, a pilot study was performed on isolated human islets to investigate the ability to treat such infections with an antiviral compound.

It was found that one of the virus strains replicated in human β-cells without affecting their main function for at least seven days, which in vivo may increase a virus’s ability to persist in islets.

Nitric oxide was induced by synthetic dsRNA, poly(IC), but not by viral dsRNA in rat insulinoma cells in the presence of IFN-γ, suggesting that this mediator is not induced by EV infection in β-cells and that poly(IC) does not mimic an EV infection in this respect.

All three virus strains were able to induce production of the T-cell chemoattractant interferon-γ-inducible protein 10 (IP-10) during infection of human islets, suggesting that an EV infection of the islets might trigger insulitis in vivo.

Antiviral treatment was feasible in human islets, but one strain was resistant to the antiviral compound used in this study.

To conclude, a potential mechanism is suggested for the involvement of EV infections in T1D. If EV infections induce IP-10 production in human islet cells in vivo, they might recruit immune cells to the islets. Together with viral persistence and/or virus-induced β-cell damage, this might trigger further immune-mediated β-cell destruction in vivo.

Keywords: enterovirus, type 1 diabetes, β-cells, human pancreatic islets, picornavirus, chemokines, nitric oxide

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List of Publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I Complete Nucleotide Sequence of a Coxsackievirus B-4 strain Capable of Establishing Persistent Infection in Human Pancreatic Islet Cells: Effects on Insulin Release, Proinsulin Synthesis, and Cell Morphology
Hong Yin, Anna-Karin Berg, Jan Westman, Claes Hellerström, Gun Frisk

II dsRNA formed as an intermediate during Coxsackievirus infection does not induce NO production in a β-cell line with or without addition of IFN-γ
Anna-Karin Berg, Asma Elshebani, Arne Andersson, Gun Frisk

III Antiviral Treatment of Coxsackie B Virus Infection in Human Pancreatic Islets
Anna-Karin Berg, Annika Olsson, Olle Korsgren, Gun Frisk
(submitted)

IV Enterovirus Infection Induces IP-10/CXCL10 Expression and Secretion from Human Pancreatic Islets in vitro
Anna-Karin Berg, Olle Korsgren, Gun Frisk
(manuscript)
Abbreviations

ANOVA: analysis of variance
ATCC: American Type Culture Collection
CAR: coxsackie- and adenovirus receptor
CAV: Coxsackie A virus
CBV: Coxsackie B virus
CD: cluster of differentiation antigen
cDNA: complementary deoxyribonucleic acid
CPE: cytopathic effects
DAF: decay accelerating factor
DMSO: dimethyl sulfoxide
DNA: deoxyribonucleic acid
dNTP: deoxyribonucleotide triphosphate
dpi: days post infection
dsRNA: double-stranded RNA
eIF: eukaryotic initiation factor
ELISA: enzyme-linked immunosorbent assay
EMEM: Eagle’s Minimum Essential Medium
ER: endoplasmic reticulum
EV: enterovirus
GAD65: glutamic acid decarboxylase 65
GMK: green monkey kidney cell line
h: hour/hours
HLA: human leukocyte antigen
IA-2: islet cell antigen-2 (Tyrosine Phosphatase)
ICA: islet cell autoantibodies
ICAM-1: intercellular adhesion molecule-1
iNOS: inducible nitric oxide synthase
IRES: internal ribosomal entry site
IFN: interferon
Ig: immunoglobulin
IL: interleukin
IP-10: interferon-γ-inducible protein 10
MHC: major histocompatibility complex
mRNA: messenger RNA
NF-κB: nuclear factor-κB
NO: nitric oxide
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>NOD</td>
<td>non-obese diabetic</td>
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<td>NTR</td>
<td>non-translated region</td>
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<td>PCR</td>
<td>polymerase chain reaction</td>
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<td>p.i.</td>
<td>post infection</td>
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<td>PKR</td>
<td>dsRNA-dependent protein kinase</td>
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<tr>
<td>Poly(IC)</td>
<td>polyinosinic-polycytidylic acid</td>
</tr>
<tr>
<td>RCC</td>
<td>RINm5F cell clusters</td>
</tr>
<tr>
<td>RIG-I</td>
<td>retinoic acid-inducible gene I</td>
</tr>
<tr>
<td>RINm5F</td>
<td>rat insulinoma m5F cell line</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>RT</td>
<td>reverse transcription</td>
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<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
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<tr>
<td>T1D</td>
<td>type 1 diabetes</td>
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<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>tissue culture infectious dose-50</td>
</tr>
<tr>
<td></td>
<td>the reciprocal of the highest dilution of a virus sample</td>
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<td></td>
<td>that causes CPE in 50% of inoculated cell cultures</td>
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<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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<td>VP</td>
<td>viral protein</td>
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Introduction

Type 1 diabetes (T1D) is a multifactorial disease which leads to β-cell loss and insulin deficiency. Genetic factors predispose to T1D but environmental factors such as virus infections have been suggested to contribute to the disease as triggers or precipitators of β-cell death. This study focuses on the potential involvement of EV infections in T1D pathogenesis, since these are the most common viruses associated with the disease.

One proposed mechanism for the involvement of EV in T1D is that the virus triggers β-cell destruction by infecting these cells. This could lead to direct damage caused by the virus infection or indirect damage via an activation of the immune system. To evaluate if EV have a causal role in T1D pathogenesis, the effects of such infections on human islets must be studied.

This thesis summarises studies of three different Coxsackie B4 virus (CBV-4) strains which can infect human islets: the effects of such infections on islet cells, particularly on the β-cells, and a pilot study of treatment of in vitro EV infections in human islets with an antiviral compound.
Background

Type 1 diabetes

History
Diabetes mellitus was described more than two thousand years ago as a fatal disease that caused wasting away of the body, extreme thirst and frequent urination. The Indian physician Sushruta described the sweet taste of urine from the affected individuals already in 400 B.C.. It was however long before a group of scientists in Toronto, Canada in the early 1920’s would discover that these symptoms were caused by a deficiency in the glucose regulatory hormone, insulin [1].

Today it is known that the lack of insulin in individuals with type 1 diabetes (T1D) is due to a selective loss of the body’s insulin-producing cells, the β-cells of the pancreatic islets. The process responsible for the loss of the β-cells is, however, not completely understood.

Clinical Features and Epidemiology
Type 1 diabetes, also known as insulin-dependent diabetes mellitus, usually appears in childhood or adolescence, and is in 70% of cases accompanied by autoantibodies directed against cytoplasmic islet cell antigens (ICA), insulin, glutamic acid decarboxylase (GAD$_{65}$) or tyrosine phosphatase (IA-2) [2]. The clinical symptoms thirst, weight loss and polyuria (secondary to hyperglycaemia) develop acutely, but antibodies against one or more of the above mentioned islet autoantigens can in most cases be detected in the circulation months or even years before diagnosis, suggesting that β-cell destruction takes place over a longer period of time. Autoantibodies are not believed to be directly involved in the destruction of the β-cells, but have been interpreted as markers for gradual destruction of the β-cells and can be used to predict disease in non-diabetic individuals [2]. It is estimated that around 80–90% of the β-cell mass has been lost by the time clinical symptoms appear [3].

The incidence of T1D varies between countries and different ethnic groups but seems to be increasing worldwide, particularly in low incidence countries [4] and in children below the age of five [5]. Finland and Sardinia have the highest incidence rates in the world, with close to 50 new T1D
cases per year per 100 000 children aged 0–14 years in Finland [6] and 37/100 000 per year in Sardinia [7]. China has the lowest incidence rate at 0.1/100 000 per year in this age group [8]. In Sweden, 27/100 000 children are diagnosed with T1D every year [8]. The frequency of disease onset has also been observed to vary between seasons and is most common in the autumn which suggests an infectious etiology [9].

Insulitis
Pancreata have been obtained post mortem from patients who died shortly after they were diagnosed with T1D. Histological study of these revealed that many islets did not contain insulin and appeared to have shrunk, presumably due to the loss of β-cells [10]. The exocrine tissue around the shrunken islets also appeared atrophied [10]. In the same pancreata, islets that contained β-cells were also found and some of these islets were infiltrated by immune cells, a condition termed ‘insulitis’ [10-12]. The immune cell infiltrate was composed mainly of interferon (IFN)-γ-containing lymphocytes but also included some macrophages [12]. Insulitis was found in the majority of the patients with recently diagnosed T1D and in a few cases in patients who had been diagnosed with the disease several years earlier [11]. It was however almost never present in islets without β-cells [10, 11], suggesting that these cells were the target [10].

Immune-Mediated Destruction of β-Cells
Due to the presence of immune cells in the islets and islet cell specific autoantibodies in the blood of T1D patients, T1D is believed to be an autoimmune disease. Consequently, much research has been focused on the possible immune-mediated mechanisms of β-cell destruction.

Lymphocytes
T-lymphocytes, both CD4+ and CD8+, have been shown to be important effectors of β-cell destruction in the non-obese diabetic (NOD) mouse and transfer of autoreactive T-cells from a diabetic mouse to a non-diabetic mouse is sufficient to induce diabetes [13]. The NOD mouse model mimics many aspects of the human disease including the presence of autoantibodies and insulitis [13].

Autoantibodies are not destructive to β-cells and are therefore not believed to play a part in the effector phase of β-cell destruction [13]. In support of this, T1D has occurred in a patient lacking B-cells which shows that antibodies are not an absolute requirement for the disease to develop [14]. B-cells could however play a part as antigen presenting cells during the activation of T cells.
Cytokines and Nitric Oxide
Proinflammatory cytokines and other soluble mediators secreted by infiltrating T-cells, NK-cells and macrophages might also trigger signalling pathways that impair β-cell function and lead to β-cell death. T-cells produce tumor necrosis factor-α (TNF-α) and IFN-γ which can induce apoptosis in rodent β-cells [15]. Macrophages produce interleukin-1 (IL-1) as well as TNF-α and are major producers of the free radical nitric oxide (NO) which can induce both apoptosis and necrosis in rodent β-cells [16]. Both macrophages and IFN-γ-containing lymphocytes have been detected in the cellular infiltrate of islets from T1D patients [12]. The importance of cytokine-induced β-cell death in human disease is however uncertain. Studies of isolated islets have shown that human islets are more resistant than rodent islets to the toxic effects of cytokines [17] and NO [18] and that human β-cell function was impaired only after exposure to combinations of two or more of the cytokines [17].

In addition to directly inducing β-cell death, exposure to cytokines may prime the cells for destruction by upregulating major histocompatibility complex (MHC) molecules [19] or Fas receptor [15] on the surface of the β-cell which could increase their susceptibility to T-cell-mediated killing.

An Initiating Factor
Several components appear to be necessary for the initiation of autoimmune β-cell destruction. Antigen presenting cells (mainly macrophages and dendritic cells) are needed in the vicinity of the islets for the activation of T cells [20]. In addition, something must trigger the initial tissue damage that releases the β-cell antigens and create the right environment of cytokines and chemokines (T helper 1 type) to recruit and activate destructive autoreactive immune cells.

Direct Damage to β-Cells
An alternative mechanism to immune-mediated β-cell killing is direct damage to β-cells caused by exogenous factors. In addition to causing some β-cell death this could be the first insult to the islets that releases antigens and triggers immune-mediated mechanisms and autoimmune reactivity. In mice, multiple low doses of the toxin streptozotocin can be used to cause the first damage to the β-cells which then stimulates further immune-mediated β-cell killing [21]. Viruses with lytic capability and tropism for the β-cell might also play this role.
Genetic Factors

The susceptibility to develop T1D is inherited and has been linked to more than 20 different chromosome regions [22]. Among these, the IDDM1 region on chromosome 6, containing the human leukocyte antigen (HLA) genes provides the single largest genetic risk [22]. In particular the HLA class II alleles DRB1*03, DRB1*0401 and DQB1*0302 have been identified as the primary risk factors and almost 90% of diabetic children have at least one of these risk alleles [23]. To complicate matters, the DR15-DQA1*0102-DQB1*0602 haplotype can protect against disease in an individual who carries one of the risk alleles [24]. HLA class II molecules are involved in the presentation of antigen to immune cells during the induction of tolerance to self antigens as well as during the activation of the immune system and it has been suggested that their role in diabetes may be related to this function. Hypothetically, individuals carrying high risk alleles might be less efficient at eliminating self-reactive immune cells during the selection process in the thymus which determines the T cell repertoire and hence be more prone to autoreactivity. However, it is also possible that the HLA haplotypes identified as high risk are not directly involved in disease pathogenesis but are instead markers for other genes.

Environmental Factors

Despite the identification of genetic risk factors, 90% of those who develop T1D do not have a first degree relative with the disease [25] and the concordance rate of T1D between identical twin pairs is less than 50% [26, 27]. This indicates that environmental factors are also important determinants of disease development.

Many different environmental factors have been suggested to be involved: virus infections, toxins, dietary factors, growth (leading to increased insulin requirements), ante- and perinatal risk factors and stressful life events. These may act alone or in different combinations in individuals with different genetic risk, and may act as initiators or as enhancers during different stages of the β-cell destructive process [28]. Among the dietary factors, exposure to cow’s milk proteins early in infancy has received the most attention [29]. Nitrate, nitrite and nitrosamines present in food and water and bafilomycin A1, a product of the soil bacteria Streptomyces which can contaminate vegetables such as potatoes and beet, are possible environmental toxins which might cause β-cell damage [28]. Viruses are likely environmental factors because they induce strong immune responses and can be tropic for the pancreas or islets.

Several viruses have shown association with T1D: EV [30], congenital rubella [31], rotavirus [32, 33] cytomegalovirus, Epstein-Barr virus, varicella zoster virus, retrovirus and mumps virus (reviewed in [34]). However, only
congenital rubella [35] has shown evidence that suggests a causal relationship. Rubella is now rare since the introduction of vaccines but EV are common infections that have been strongly associated with T1D [30]. It is not likely that all EV infections will lead to β-cell destruction and T1D, since T1D affects only a small proportion of all those who encounter infections with these viruses. Most likely, T1D is the result of a combination of several factors: the virus strain, the genetic susceptibility of the host and the timing of the infection(s).

Enteroviruses
Enterovirus Infections
EV infections occur frequently in the population, particularly among young children [36], and often without symptoms [37]. The infections are most common in late summer and autumn and less frequent during the rest of the year [37]. As the name implies their normal site of replication is in the gut. The EV are stable even at very low pH which allows them to survive the passage through the stomach to infect the intestinal tract. The virus is shed for one to two months after infection [38] and sometimes for as long as six months [39, 40] and is mainly spread through contact with faeces or through contaminated food and water. The virus can also replicate in the upper respiratory tract and is then shed for one to three weeks after infection. The stability of the virus allows it to remain infectious for a long time in food and water. It can resist freezing and thawing but is inactivated by heat or drying [41].

Although the majority of EV infections pass by unnoticed, in some cases there are mild symptoms (fever, rashes, common cold symptoms or mild diarrhoea) and if the virus spreads from the gut via the blood and lymphatic system to other organs some EV can cause more serious diseases [41]. In perspective, it has been estimated that only 1% of poliovirus infections leads to neurological symptoms, suggesting that the ability to cause severe disease is not really a necessary (or beneficial) part of the virus life cycle but is more of an accidental event [42]. Besides poliomyelitis, EV are known to cause myocarditis, paralysis, a majority of aseptic meningitis cases and 10–20% of viral encephalitis cases [36, 41]. In neonates, infections can be very serious due to the involvement of multiple organs, and can even be fatal [36], and maternal infections during pregnancy have been associated with spontaneous abortion [43].
Classification of Enteroviruses

The Picornavirus Family

The EV belong to the picornavirus family, which includes a number of important human and animal pathogens e.g. the rhinoviruses (common cold viruses), human Hepatitis A virus (a common contaminant of shellfish which causes diarrhoea or jaundice) and foot-and-mouth disease virus (causing severe disease in cattle). The picornaviruses are divided into nine genera, the Enteroviruses, Hepatoviruses, Cardioviruses, Rhinoviruses, Aphthoviruses, Parechoviruses, Erboviruses, Kobuviruses and Teschoviruses [44], which are all non-enveloped single-stranded RNA viruses that cannot be distinguished morphologically by electron microscopy. The name picorna describes their small size (28-30 nm) and their RNA genome (pico=a small unit of measurement, 10^-12) [45]. Recent comparison of rhinovirus and EV sequences has shown that these two groups are more closely related than previously thought [46] and the latest suggestion is that they should be reclassified as one genus [47].

The Enterovirus Genus

The EV are a group of viruses with similar characteristics: they all share the enteric route of infection and the ability to resist inactivation at low pH [48]. Of note, rhinoviruses, although genetically similar to EV, do not have these properties and an acid sensitivity test can be used to differentiate between the two groups. The most well-known EV member is poliovirus, known as the cause of the epidemics of infantile paralysis (poliomyelitis) in the middle of the 20th century. The other members are the coxsackie A and B viruses (CAV, CBV), named after the town of Coxsackie in New York state where they were first isolated, the echoviruses (enteric human cytopathogenic orphan viruses) and the more recently identified EV which have been consecutively numbered from 68 to 97. New EV are still being discovered [47]. The original subdivision of coxsackieviruses into subgroups A and B, was based on the type of paralysis they were able to induce in suckling mice: flaccid paralysis (type A) or spastic paralysis (type B) [49]. Since the sequencing of many EV genomes, a new classification based on genetic similarity has been suggested [44]. According to this, the human EV are reclassified into five species and the coxsackie B viruses are included in species B (Table 1). Some of the more recently identified EV serotypes have also been added to these groups [50-53].
Table 1. The human enteroviruses

<table>
<thead>
<tr>
<th>Species</th>
<th>Serotypes</th>
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<tbody>
<tr>
<td>Poliovirus</td>
<td>Poliovirus 1-3</td>
</tr>
<tr>
<td>Human Enterovirus A</td>
<td>Human coxsackievirus A2-A8, A10, A12, A14, A16, Human enterovirus 71, 76, 89, 90, 91</td>
</tr>
<tr>
<td>Human Enterovirus B</td>
<td>Human coxsackievirus B1-B6, A9, Human echovirus 1-7, 9, 11-21, 24-27,29-33, Human enterovirus 69, 73, 74, 75, 77, 78</td>
</tr>
<tr>
<td>Human Enterovirus C</td>
<td>Human coxsackievirus A1, A11, A13, A15, A17-22, A24</td>
</tr>
<tr>
<td>Human Enterovirus D</td>
<td>Human enterovirus 68, 70</td>
</tr>
</tbody>
</table>

Serotypes and Strains

The different species of EV are further subdivided into serotypes, which by definition are all the viruses which are neutralised or rendered uninfectious by treatment with a type specific serum. Cell cultures can be used as indicator systems for virus neutralisation tests. The Coxsackie B viruses are divided into six different serotypes (CBV-1–6). Within a single serotype there can be many strains of virus with different characteristics which arise during normal infections due to the rapid mutation rates of the viral RNA genome [54].

Enterovirus Association with Type 1 Diabetes

The first studies suggesting that EV could be involved in T1D pathogenesis reported a correlation between the seasonal variation of diabetes onset and the prevalence of EV infections, particularly CBV-4 [9]. Diabetes patients were also found to have antibodies against CBV-4 more frequently than controls [55]. Since then several studies have shown a higher frequency of IgM antibodies against CBV [56, 57] or EV-RNA [58-60] in the blood of recently diagnosed diabetes patients compared to controls suggesting that EV infections occur frequently close to the time of diabetes onset.

Several prospective studies following children with increased genetic risk for T1D have also presented evidence of increased frequency of EV infections in those who later developed T1D [61, 62]. The EV infections occurred both close in time to disease onset and several years before [61]. Moreover, the infections were often associated in time with the appearance of autoantibodies [61-65] suggesting an induction of β-cell damage during the infections. Although such studies do not prove a causal association between EV
and T1D, CBV isolated from patients have in two cases been able to induce diabetes [66] or glucose intolerance [67] in mice.

Pancreata examined from patients with T1D have shown IFN-α expression and hyperexpression of MHC I in residual β-cells in 33 of 34 patients [68], which is compatible with the hypothesis that a virus infection of the β-cells is involved in T1D. Recently, EV RNA was also detected by in situ hybridisation exclusively in the islets of a few T1D patients [69]. Altogether this suggests that EV infection of the β-cells might be part of the initiation of T1D development or be the last stress that causes clinical symptoms.

Potential Mechanisms of T1D Induced by a Virus Infection

Based on the evidence from patients, EV infections could play a role at several different stages of T1D development from disease initiation to the precipitation of clinical onset and possibly several infections are required. Both direct virus-induced effects and immune-mediated mechanisms have been proposed to explain how a virus infection might be involved in the destruction of β-cells in T1D.

Direct Damage

A virus might cause direct β-cell damage through virus-induced cell lysis. In support of this, EV can infect human β-cells in vitro and most EV strains have been shown to induce cell lysis in isolated human islets in culture [70, 71]. A series of lytic infections might lead to a gradual loss of β-cells that finally presents as T1D. A lytic infection might also lead to the initial release of β-cell antigens necessary for the activation of autoimmune responses.

Induction of Immune-Mediated Destruction

If an EV epitope is sufficiently similar to a β-cell antigen a virus infection might induce a crossreactive immune response by molecular mimicry which could lead to β-cell destruction. This mechanism would not require virus infection of the islets. In the case of EV, homology has been found between the CBV protein P2C and the autoantigen GAD65 [72] and also between enteroviral capsid proteins and heat shock protein 60 [73], but the evidence for the involvement of this mechanism in T1D is inconclusive [74].

If the islets (including the β-cells) are infected by the virus, as suggested by the detection of EV-RNA in the islets of some T1D patients [69], some β-cell destruction might also be caused by an immune response directed against virus antigens. The fact that EV-RNA has been detected in islets in association with insulitis in infants who had died of fulminant EV infections [69] suggests that this could be a possible mechanism. Virus-induced immunopathology would also be compatible with the insulitis found in most pancreata from recently diagnosed T1D patients. If the virus is able to persist in β-cells it might cause a chronic inflammation which could explain the
slowly progressing β-cell destruction. Alternatively, several acute infections might induce gradual immune-mediated β-cell damage in analogy to the lytic infections discussed above.

A virus infection of the islets might also induce an autoimmune reaction by acting as an adjuvant for the activation of immune responses against local self-antigens. Proinflammatory cytokines induced by the virus infection and direct recognition of viral dsRNA could activate antigen presenting cells [75] and upregulate HLA class II molecules, promoting the development of an antigen-specific immune response [76]. In combination with a virus-induced release of β-cell antigens this might lead to activation of β-cell-autoreactive immune cells. The ability of a CBV-4 infection of the β-cells to activate pre-existing autoreactive T-cells through the release of β-cell antigens and the induction of an inflammatory response has been shown in the BDC2.5 transgenic mouse [77, 78].

To increase our understanding of how EV infections might lead to T1D we must know more about the effects of EV infections on the pancreatic β-cells.

The Cellular Life Cycle of Enteroviruses

The Virion

The EV particle consists of a simple non-enveloped protein shell surrounding a single copy of the (+)stranded RNA genome bound to a viral protein, VPg. The virus capsid is composed of 60 copies of each of the four capsid proteins (VP1–4) arranged with icosahedral symmetry (Fig. 1). VP1–3 face the outer surface and VP4 is located on the inside of the capsid [45]. The genome is a ~7400 nucleotide long molecule of RNA containing a single coding region flanked by non-coding regions (NTRs) at the 5’ and 3’ends and a polyA-tail at the 3’end [41].

Figure 1. Schematic view of an EV virion
Attachment and Entry

The cellular life cycle of the virus (Fig. 2) begins with attachment to appropriate receptors on the host cell and entry of the virus (or the RNA genome) into the cell’s cytoplasm. There is no consensus on the mechanism of entry which seems to proceed by different mechanisms depending on the virus or on the receptor used in a particular cell type. Binding to some receptors induces a conformational change in the capsid [79] which releases the RNA, a process termed uncoating, and allows the RNA to enter into the cytoplasm while leaving the empty capsid outside the cell. In other cases the whole virus particle may be taken up by the cell by endocytosis before uncoating occurs [80].

Many molecules are used as receptors. The most important receptor used by the CBV is the coxsackievirus and adenovirus receptor (CAR), a member of the immunoglobulin superfamily. CAR is a common receptor for all six serotypes of CBV and is also used by some adenoviruses [81]. CAR is used for attachment to the cell as well as for uncoating [79] and entry [82]. The decay accelerating factor (DAF/CD55) is another receptor molecule that can be used by some CBV, at least for attachment [83]. A third receptor proposed for the CBV is a 100 kDa protein that is a member of the nucleolin family [84]. The intercellular adhesion molecule-1 (ICAM-1) is used by some CAV and by rhinoviruses [85] and it may be used by some strains of CBV-4 as suggested by antibody blocking experiments [86]. Polioviruses use the poliovirus receptor, and the integrin family can be used by several of the echoviruses [87-89] and by CAV-9 [90]. Heparan sulphate can bind echovirus-6 [91] and CBV-3 [92]. Antibody blocking experiments performed on human islets, suggest that integrin-α,β3 [69, 93], the poliovirus receptor [69] and CAR [69, 94] are used as receptors by different EV on the β-cells.

Translation

After entry, the viral genomic RNA functions as an mRNA and is translated into a single polyprotein by host cell ribosomes. The polyprotein, which includes several proteases (2A, 3C and 3CD), then cleaves itself into the separate proteins necessary for replication, including the capsid proteins and an RNA-dependent RNA-polymerase (3D\textsuperscript{pol}) [45]. The virus also encodes other non-structural proteins (2BC, 2B, 2C, 3AB, 3A, 3B (=VPg)) which are used in different steps of the replication and to modify host cell functions to accommodate virus replication. Despite the translation of all proteins at equimolar amounts the virus can regulate the relative activity of different proteins to some degree by fast and slow proteolytic cleavage by the three proteases. More efficient cleavage at some sites leads to higher amounts of the some proteins while others are to a higher degree left as precursors [42].
**Virus Replication**

Replication of the virus genome takes place in the cytoplasm and is organised in replication complexes on membranes formed from the endoplasmic reticulum ER [95, 96]. The virus-encoded RNA-dependent RNA-polymerase (3Dpol) first makes a minus strand RNA copy of the genome as a template and then uses this to make multiple copies of the plus strand genome. During virus replication double-stranded RNA (dsRNA) is therefore present as an intermediate form [45].

**Virion Assembly and Release**

The virus-encoded capsid proteins VP1, VP3 and VP0 (a precursor for VP2 and VP4) form protomers and 60 protomers assemble into a capsid which packages the RNA genome [45]. The virions then mature into infectious virus particles by cleavage of the VP0 protein in the capsid into VP2 and VP4 [45]. The newly formed virus particles are finally released from the cell by cell lysis [45] or by alternative non-lytic mechanisms not yet identified [97].

![Figure 2. The replication cycle of EV](image)

**Effects of EV Replication on the Host Cell**

Since the virus is dependent on host cell factors for its replication, it has evolved efficient ways to redirect the cell’s activities to promote replication of the virus and also to inhibit some of the antiviral responses of the host cell. In doing this, the virus often disturbs normal cellular function and can
ultimately cause cell death. Most of these effects have been studied with poliovirus. The extent of effects on the host cell, however, varies between different EV serotypes and virus strains as is exemplified below.

**Host Cell Translational Shut-Off**

Translation of the polyprotein encoded by the EV RNA is initiated via an internal ribosomal entry site (IRES) located in the 5’NTR and is therefore independent of the normal initiation factors required for the translation of the host cell’s capped mRNA (cap-dependent translation). Some EV use this difference to redirect ribosome activity from the host cell’s mRNA to the virus’s RNA. By cleaving one or more of the initiation factors necessary for cellular cap-dependent translation, these viruses shut off the host cell’s protein synthesis, **host cell shut-off** [98, 99], while IRES-mediated translation of the viral protein can proceed with less competition. The viral protease 2A and possibly protease 3C are believed to be responsible for this effect [99]. Not all cellular proteins are affected by the host cell shut-off. Some genes, e.g. the stress-response gene heat shock protein 70 [100] and the translation initiation factors eIF4GI [101, 102] and eIF4GII [102] can use cap-independent, IRES-mechanisms of translation. Shut-off of host cell translation is not required for EV replication [87].

**Rearrangement of Membranes during EV Replication**

EV replication takes place on intracellular membranes probably derived from the ER [95, 96]. This can be seen as a rearrangement of membranes and an accumulation of host cell derived vesicles [95] that colocalise with newly formed viral RNA in infected cells [96]. The formation of vesicles starts at the onset of virus translation [96] and is probably a combined effect of viral proteins 2BC and 3A [95]. The virus also uses the cell’s microtubuli system to move the vesicles and viral RNA closer to the nucleus during replication [96, 103] but this migration is not necessary for the production of virus [96].

**Effects on Intracellular Trafficking of Vesicles**

Another function related to the effect on host cell vesicles has been discovered in EV protein 3A. When this protein is expressed alone in cells it can block the intracellular transport of vesicles from the ER to the Golgi [104, 105]. This can have two major effects on the cell: 1) the secretion of proteins from the cell is blocked which can inhibit secretion of cytokines such as IFNs, and stop the infected cell from signalling for help [106]; 2) the renewal of proteins to the cell membrane is blocked which can lead to a down-regulation of cell surface receptors with a high turnover rate such as the TNF-α receptor [107, 108]. This in turn might reduce the cell’s susceptibility to apoptosis induction via external signals. The ability of 3A protein to block host cell secretion is not essential for the virus life cycle **in vitro** and has only
been found in some EV (poliovirus and CBV-3) [104, 105]. However, this function may be of importance in vivo since it might allow the virus to evade the immune system.

**Ca^{2+} Effects**

It has been demonstrated that Coxsackieviral protein 2B can deplete intracellular stores of Ca^{2+} and increase the influx of extracellular Ca^{2+} and that this might be a way for the virus to induce an anti-apoptotic state in the cell [109]. In the β-cell an effect on Ca^{2+} regulation might also affect the signals leading to insulin release. An inhibitory effect of CBV-4 infection on the Ca^{2+} influx after glucose stimulation has been shown in EV-infected mouse β-cells [110].

**Inactivation of Transcription Factors**

Poliovirus protease 3C can cleave and inactivate several different transcription factors, the TATA-box binding protein [111], cyclic AMP-responsive element binding protein [112], p65-RelA component of the NF-κB complex (inactivating NF-κB) [113] and thereby downregulate the expression of cellular genes in infected cells. This might be a viral strategy to inhibit transcription of antiviral response genes in the host cell.

**Cytopathic Effects and Cell Death**

In common cell lines used for propagation or isolation of EV, such as green monkey kidney (GMK) cells, virus replication usually induces characteristic morphological changes to the cells known as cytopathic effects (CPE). The typical EV-induced CPE is characterised by rounding of the cells which make them refractory to light, followed by cell death and detachment of the cells from the surface. It is not known exactly what causes CPE.

Not all productive infections induce cytopathic effects. The virus may replicate without CPE if for example only a small fraction of the cells are infected at one time, a carrier state persistent infection [114]. The virus can also establish a steady state persistent infection in a large number of cells and continuously produce high amounts of infectious virus without CPE by unknown mechanisms [97, 115]. Lastly the virus may be able to persist in cells for some time without replication, possibly in a stable dsRNA conformation, which has been found in association with chronic inflammatory myopathy in vivo [114].

**Antiviral Response of the Host Cell**

The production of dsRNA and viral proteins during viral replication triggers an antiviral response in the host cell. Cells can detect dsRNA by several established pathways: dsRNA-dependent protein kinase (PKR), 2′,5′ oligoadenylate synthetase and Toll-like receptor-3 (TLR-3). PKR induces a block of protein translation in the cell in an attempt to limit virus production
The enzyme 2',5' oligoadenylate synthetase activates RNaseL which breaks down single-stranded RNA in the cell [116]. TLR-3 triggers activation of transcription factors (including NF-κB) [117] that are important for expression of antiviral response genes such as iNOS [118-120], IFNs and other cytokines [117]. TLR-3 expression has been detected in mouse and human islet cells [75]. Recently, a novel intracellular receptor for dsRNA was discovered, the retinoic acid-inducible gene I (RIG-I), which activates transcription of similar antiviral response genes as TLR-3 [117]. NF-κB can also be activated in the absence of dsRNA after the binding of viral capsid proteins to cell surface receptors, at least in monocytes [121].

Expression of the iNOS protein leads to increased formation of NO which can have direct antiviral effects. It has been shown that exogenously added NO or NO produced by activated macrophages can reduce CBV-3 replication via the inactivation of viral protease 3C [122]. Data suggest that β-cells are also capable of NO production in response to dsRNA [123, 124]. It has been shown that the addition of the artificial dsRNA molecule poly-inosinic-poly-cytidylic acid (poly(IC)) to the cells together with IFN-γ can induce expression of the iNOS gene in purified rat β-cells [123, 124] and NO production in isolated islets from rats [120, 123] as well as humans [120, 124].

It is not clear which of these antiviral response pathways, if any, are activated during EV infection of β-cells. In addition, some picornaviruses are able to counteract several antiviral signalling pathways triggered by dsRNA [125].

**EV Infections in Isolated Human Islets**

While much is known about the cellular effects of EV infections on primate or human cell lines frequently used for EV propagation, the effects of EV infection on human islet cells and β-cells have hardly been studied at all. This is mainly due to the low availability of human islet tissue for research. The studies that have been performed have demonstrated the capacity of different EV serotypes to infect, damage and ultimately kill human β-cells under *in vitro* conditions [70, 71]. Under similar *in vitro* conditions some strains from the same EV serotypes can replicate with little or no islet-destructive effects [70, 71, 126] which demonstrates that the effects of EV infection on human islets can differ greatly between different virus strains of the same serotype [70, 126]. Different culture conditions may also promote islet production of IFN-α which has been associated with persistent infections with some EV strains [94]. Islet cells have also been shown to upregulate mRNA coding for proinflammatory cytokines and chemokines after infection with different EV [127, 128], suggesting that EV infection might additionally promote immune-mediated mechanisms of β-cell destruction.

In patient studies many different serotypes of EV have been associated with T1D, suggesting that if there is a certain property that makes a virus
diabetogenic, this property is not linked to a certain serotype but may be present within several serotypes [59, 129]. To understand the range of possible effects that EV infection can have on human islet cells and the potential mechanisms that could lead to T1D, different virus strains with different characteristics should be studied.

Antiviral Strategy to Prevent T1D
If EV infections are truly causal agents of β-cell destruction in T1D, there may be a possibility to reduce the damage by inhibiting the replication of EV in patients. Antiviral treatment at diagnosis of T1D might halt the progression of the disease and save the remaining β-cells. Vaccination might not be efficient if a wide variety of EV can cause the disease, but could be used if a particular diabetogenic strain or a common epitope is discovered. An alternative strategy could be to use an antiviral drug with broad specificity. Pleconaril is the latest of the WIN-compound family of capsid-binding molecules which can inhibit uncoating of the virus [130, 131] and/or attachment to picornaviral receptors on the host cell [132]. Pleconaril has been shown to block the replication of most EV [133] and rhinovirus serotypes [134] in vitro and has been used in clinical trials to treat common colds, aseptic meningitis and neonatal infections [135-137]. No anti-enteroviral agents have yet been approved for clinical use.
Aims of the Study

General Aim

The overall aim of this thesis was to explore potential mechanisms that might be involved in the pathogenesis of T1D through the study of EV infections in islets and β-cells.

Specific Aims

The specific aims of the thesis were:

- To study a persistent CBV-4 infection of human pancreatic islets: to sequence and compare the genome of this virus strain to other CBV and to study the effects of this infection on host cell function and morphology (I)
- To evaluate the RINm5F cell line as a model for EV infection of β-cells (II)
- To investigate if the cytotoxic mediator NO is produced in virus-infected RINm5F cells (II)
- To test if antiviral treatment of CBV-4 infection in human islets is feasible (III)
- To measure if the chemokine IP-10 is induced in and secreted from human islets during infection with different strains of CBV-4 (IV)
Materials and Methods

More detailed descriptions of all methods can be found in the original publications.

Cell Culture
All cells were cultured at 37°C, in a humidified atmosphere of 5% CO₂ in air.

Human Islets (I, III, IV)
Human pancreatic islets from heart-beating organ donors were isolated at the Central Unit of β-Cell Transplant, Brussels (I), or at the Department of Oncology, Radiology and Clinical Immunology at Uppsala University (III, IV). The islets were cultured free-floating in RPMI 1640 containing 5.5 mM glucose and 10% foetal or newborn bovine serum, benzylpenicillin (100 U/ml), and streptomycin (0.1 mg/ml). For each experiment islets were identified under a microscope and handpicked from the residual exocrine tissue. Virus infections and the respective control treatments were always performed using islets from the same donor.

Insulin-Producing Cell Line (II)
Rat insulinoma-derived RINm5F cells (kindly provided by Prof. Claes Hel-lerström) is an insulin-producing cell line that has been used previously in studies of the role of NO production as a mediator of cytokine-induced β-cell damage [138-140]. These cells were cultured as monolayer cultures or as free-floating RINm5F cell clusters (RCC) in Eagle’s Minimum Essential Medium (EMEM) supplemented with 10% newborn bovine serum. The RCC originated from some of the monolayer cultures (Paper II Figure 1a) where mushroom-like cell clusters formed and detached and could subsequently be maintained free-floating in culture (Paper II Figure 1b). The RCC were harvested from cell culture flasks and transferred to non-attach six-well plates where they were cultured for 3–7 days before the virus inoculations.
GMK Cells (I-IV)
Green Monkey Kidney (GMK) cells were used for virus propagation and
virus titrations. They were cultured in EMEM supplemented with 10% new-
born bovine serum.

Viruses
Viruses Strains (I–IV)
The following virus strains were used in this study: the CBV-4 prototype
strain JVB (ATCC) (I); the CBV-4 V89-4557 strain (III, IV) which causes
islet degeneration when human islets are infected in vitro [70]; the CBV-4
VD2921 strain (I, III, IV) which causes a non-lytic infection in isolated hu-
man islets in culture [70]; the CBV-4 E2 strain (kindly provided by Prof.
Tapani Hovi) (II, IV). The V89 4557 strain and the VD2921 strain are both
plaque-purified strains originally isolated from patients with aseptic menin-
gitis. The E2 strain has previously been shown to infect islet cells in mice
and cause β-cell degranulation [141] and been shown to infect and lyse hu-
man islet cells in culture [70]. These CBV-4 strains have previously been
used for the study of virus involvement in type 1 diabetes in patients [57,
142-144] and in mice [145] and characterised in cell culture [86, 110, 146].
In the RINm5F cell infections (II) CAV-7, CBV-1 (in-house strains), CBV-3
Nancy strain and Echovirus 7 Wallace strain (ATCC) were also used. Vi-
ruses were propagated in GMK cells and virus suspensions prepared by
freeze-thawing the infected GMK cells upon appearance of cytopathic ef-
fects (CPE) followed by centrifugation to remove cellular debris. The viruses
were diluted in serum-free EMEM to a working virus titre for infections.

Virus Inoculations (I–IV)
The human islets, RCC, or confluent RINm5F monolayer cells were inocu-
lated by addition of 10^3–10^5 tissue culture infectious doses (TCID50) of
virus to the cultures. The inoculated cells were then incubated at 37°C for 30
minutes to allow the virus to attach, after which fresh culture medium was
added to the cells.

Detection of Virus Replication (I–IV)
Virus replication was determined by TCID50 titrations on GMK cells. Sam-
ples of culture medium were aspirated from the virus-infected human islet
cultures or RINm5F cell cultures on day 0 and then at regular intervals at
least every two days until day 4 (IV), day 6 (III), day 10 (II) or day 15 (I)
post infection (p.i.). To titrate the virus content in the culture medium on the
respective days, the samples of culture medium were serially diluted and each dilution was added in triplicate to GMK cells in 96-well plates. When EV replicate in GMK cells, they induce typical morphological changes to the cells (CPE). The virus titres were determined as the reciprocal of the highest dilution able to induce CPE in 50% of the inoculated GMK cell cultures.

Antiviral Compound (III)
Pleconaril (3-(3,5-dimethyl-4((3-(3-methyl-5-isoxazolyl)propyl)oxy)phenyl)-5-trifluoromethyl-1,2,4-oxadiazole) (ViroPharma Incorporated) is a broad-reacting antiviral compound which has been shown to inhibit most enterovirus and rhinovirus strains in vitro [133, 134]. The ability of pleconaril to inhibit replication of β-cell tropic EV strains in human islets was tested using virus strains CBV-4 VD2921 and CBV-4 V89 4557. Pleconaril was dissolved in DMSO to a 200X stock solution (2 mM) and added to virus-infected human islets in culture to a final concentration of 10 μM. Two treatment strategies were compared: a) addition of pleconaril to islet cultures 30 minutes after the addition of virus; or b) addition of pleconaril to the virus inoculum 30 minutes before inoculation to allow time for pleconaril to bind to the virus before addition of the mixture to the islets. As a control, islets were infected with virus without addition of pleconaril. The virus inoculations and studies of virus replication were performed as described above.

Viral Thermostability Test (III)
The thermostability of V89 4557 virions was studied by measuring the loss of infectivity (TCID₅₀ titre) after incubation for 30 minutes at 46°C compared to incubation at 4°C.

Studies of Cell Morphology and Viability
Light Microscopy (I–III)
The human islets, RCC and RINm5F monolayer cells were examined for virus-induced morphological changes (CPE) every day under a light microscope. Islet or RCC degeneration was characterised by the loss of islet or RCC integrity, disintegration, and partial dispersion of islets or RCC. Islet destruction was graded from 0–4+, where 4+ indicated a total destruction of the islets. In addition, paraffin sections of RCC (II) were stained with hematoxylin and eosin to investigate the condition of the cells in the centres of the clusters.
Electron Microscopy of Human Islets (I)

Electron microscopy was performed on human islets infected with the VD2921 strain or the JVB strain and on uninfected controls. Infected and uninfected islets were fixed in 2% glutaraldehyde and 1% formaldehyde, followed by 1% osmium tetroxide, dehydrated in graded ethanol, and embedded in TAAB-812-resin. Ultrathin sections (500 Å) were counterstained with uranyl acetate and lead citrate.

Insulin and Proinsulin Measurements

Insulin and Proinsulin Content (I–III)

To characterise the RCC and to study the effects of virus infection on human islets, insulin (I, II, III) and proinsulin (I) contents were measured in islets and in RINm5F cells. Insulin/proinsulin was extracted from cells over night at 4°C by addition of acid ethanol (1.5 ml of 12M HCl added to 98.5 ml of 70% ethanol) and the samples were then freeze-thawed. Insulin was measured using a High Range Rat Insulin ELISA (Mercodia AB, Uppsala, Sweden) which crossreacts with human insulin. Proinsulin content was measured using a Human Proinsulin ELISA (Mercodia AB, Uppsala, Sweden).

Insulin Response During a 24-Hour Glucose Stimulation (I)

To study β-cell function in VD2921-infected islets, groups of infected and uninfected human islets were cultured for 24 hours in medium containing 16.5 mM glucose which normally stimulates insulin secretion from β-cells. For comparison, groups of infected and uninfected islets were cultured at a non-stimulatory glucose concentration (5.5 mM) for 24 hours. The insulin concentration in the culture medium was measured using a High Range Rat Insulin ELISA (Mercodia AB, Uppsala, Sweden) at the beginning and the end of the 24-hour period and the insulin release was calculated. Glucose stimulations were performed on three occasions post infection, day 3, day 7 and day 11–17.

Glucose Perfusion of Pleconaril-Treated Human Islets (III)

The β-cell function of uninfected islets which had been exposed for two–four days to 10 μM pleconaril (including 0.5% DMSO as a solvent), or to 0.5% DMSO or left untreated was tested in a dynamic perifusion system. Islets were exposed to two glucose concentrations: 1.67 mM (non-stimulatory), followed by 16.7 mM (stimulatory) and 1.67 mM again during 120 minutes. The perifusate was collected in six minute fractions and insulin concentrations analysed by insulin ELISA (Mercodia, Uppsala, Sweden).
Immunostaining for CAR on RINm5F cells (II)

Immunostaining was used to detect the CBV receptor CAR protein on RCC and on RINm5F monolayer cells. Monolayer cells were cultured on culture slides (Falcon) and RCC were attached to glass slides by addition of poly-L-lysine. The cells were fixed in acetone at 4°C and then rehydrated in PBS. A monoclonal antibody directed against CAR, RmcB (kindly provided by Dr. Michael Lindberg, University of Kalmar, Sweden), was added to the slides. The slides were washed and the binding of the antibodies visualized with a PicTure-Plus kit (Zymed Laboratories, San Francisco, CA, USA), which contains a polymer conjugate of horseradish peroxidase and Fab fragments. Potential background due to endogenous biotin activity or Fc receptors is by such means completely avoided.

RT-PCR (II, IV)

Reverse transcription and PCR were performed to detect mRNA expression of CAR (II), IP-10 (IV) and β-actin (II, IV). RNA was first isolated from uninfected RCC and from uninfected RINm5F cells (II) or from infected and uninfected human islets (IV) using RNeasy mini kit (QIAGEN). The mRNA was then reverse transcribed into cDNA using Sensiscript Reverse Transcriptase kit (Qiagen) and oligo-dT primers. PCR was performed using two microlitres of cDNA and HotStarTaq Master Mix Kit (Qiagen). The specific primers for human IP-10 [147] and for rat CAR, rat β-actin and human β-actin, designed with the online Primer-3-software programme [148], can be seen in Table 2. The PCR product was visualised on a 1.5% agarose gel containing ethidium bromide.
Table 2: PCR primers for human IP-10 and human β-actin

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Annealing temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>rat CAR (sense)</td>
<td>5’-CTCCTGGGGTTGCAATAGA-3’</td>
<td>54°C</td>
</tr>
<tr>
<td>rat CAR (antisense)</td>
<td>5’-GGGATTTTCTGGGAATCTGA-3’</td>
<td></td>
</tr>
<tr>
<td>rat β-actin (sense)</td>
<td>5’-CAGGCCATTGTAACCAACTG-3’</td>
<td>54°C</td>
</tr>
<tr>
<td>rat β-actin (antisense)</td>
<td>5’-AAGGAAAGCTGGAGAGGAGC-3’</td>
<td></td>
</tr>
<tr>
<td>human IP-10 (sense)</td>
<td>5’-CCACGTTGAGATCATTGC-3’</td>
<td>55°C</td>
</tr>
<tr>
<td>human IP-10 (antisense)</td>
<td>5’-ACATAGCACCTCAGTAGAGC-3’</td>
<td></td>
</tr>
<tr>
<td>human β-actin (sense)</td>
<td>5’-ACTGGAACGGTGAAGGTGAC-3’</td>
<td>53°C</td>
</tr>
<tr>
<td>human β-actin (antisense)</td>
<td>5’-CTCAAGTTGGGGCAAAAAA-3’</td>
<td></td>
</tr>
</tbody>
</table>

Measurements of IP-10 Protein (IV)

To study islet expression and secretion of the chemokine IP-10/CXCL-10 after EV infection, human islets were infected with different strains of CBV-4 or left uninfected and then cultured for 1–4 days. Islets and samples of the culture medium were collected for IP-10 measurements on days 0, 1, 2 and 4 p.i. both from virus-infected and uninfected cultures. Before the IP-10 measurements, both the islets and medium samples were exposed to UV light for 30 minutes to inactivate virus and the islets were then sonicated. IP-10 protein was quantified by Human IP-10 Cytoscreen ELISA (Biosource, Nivelles, Belgium).

Stimulation of Nitric Oxide Formation

Treatment of RCC with Virus, IFN and Poly(IC) (II)

DsRNA is not normally found in cells, but is a product formed during the replication of many viruses, including the EV. Previous studies have shown that if rat or human islets are exposed to dsRNA in the form of poly(IC) in the presence of IFN-γ the β-cells respond by producing NO [120, 123, 124]. The amount of NO induced by this treatment is sufficient to impair β-cell function [120, 123] and cause β-cell death at least in rodent islets [123]. To test if viral dsRNA, formed during a virus infection, would induce a similar response as poly(IC) together with IFN-γ some of the RCC infected with CBV-4 E2 were treated with rat recombinant IFN-γ (Boule Nordic AB, Sweden) 100 U/ml, which was added at 1h or 24 hrs post infection. The time-delay between addition of the virus and the IFN-γ was to allow time for formation of viral dsRNA before IFN-γ was added. As a control, artificial dsRNA, poly(IC) (Sigma-Aldrich, Sweden), was added at 100 μg/ml to some RCC together with 100U/ml rat IFN-γ or at 400 μg/ml together with 500
U/ml IFN-\(\gamma\). Since poly(IC) is already a double-stranded RNA molecule no time-delay was necessary before the addition of IFN-\(\gamma\) in these cases. RCC treated with rat recombinant IFN-\(\gamma\) alone, poly(IC) alone and a control with no additions were also included.

Nitrite Measurements (II)

In the presence of oxygen, NO forms the stable product nitrite (\(\text{NO}_2^-\)). Therefore, nitrite concentration in the culture medium was measured as an estimation of NO production. Nitrite was measured in RCC treated as described above. Samples of 100 \(\mu\)l from the culture medium taken on days 0, 1, 2, 3, 4, 5 and 7 were mixed with 10 \(\mu\)l Griess Reagent (0.5% naphthylethylenediamine dihydrochloride and 5% sulphanilamide in 25% \(\text{H}_3\text{PO}_4\)) [149]. After a colour reaction at 60°C for 2 minutes, the absorbance was read at 546 nm. Concentrations were calculated from sodium nitrite standards diluted in EMEM.

Sequencing of VD2921 (I)

Total RNA of the VD2921 strain was extracted from a virus stock with RNeasy Mini Kit (Qiagen, Qiagen Gmbh, Germany) and then stored at -70°C. Virus RNA was reverse transcribed, then denatured for 15 min at 70°C before storage on ice.

To generate templates for cycle sequencing, fragments were amplified by PCR using multiple primers (Paper I, Table I). The products were analyzed on 1% Tris-borate-EDTA agarose gels containing ethidium bromide and purified with the QIAquick PCR Purification Kit (QIAGEN Gmbh).

Amplification fragments of the VD2921 strain were sequenced in an automatic ABI Prism 310 sequencer by the Big-dye labelled terminator method. Alignment of amino acid and nucleic acid sequences was carried out using the Clustal W (1.5) multiple sequence alignment program [150]. Phylogenies of the sequences were estimated by the neighbour-joining method, using PHYLIP [151]. To examine the relationship of the VD2921 strain to other coxsackie B viruses and EV, CBV-1 to -6 were chosen, as well as a number of other EV that have been sequenced.

The positions of the amino acid changes in the structural protein of VD2921 were predicted based on the structure of the CBV-3 strain Nancy.
Results

Studies in Isolated Human Pancreatic Islets

Study I

Effects of a Persistent EV Infection on Human Pancreatic Islets
The effects of a persistent EV infection on human islet function and morphology were studied by infecting isolated human islets with the VD2921 strain of CBV-4. The appearance and function of infected islets were then studied and compared with uninfected islets during 17 days of culture.

Insulin Response to High Glucose and Insulin and Proinsulin Content
The virus replicated in the islet cells throughout the study and there was no islet destruction. The function of the β-cells was not affected by the virus infection up to 7 days p.i.. The VD2921-infected islets released similar amounts of insulin as uninfected controls when stimulated with a high glucose concentration on day 3 and day 7 p.i.. In addition, no differences were detected in the insulin or proinsulin content between the infected and uninfected islets (Table 3). At 11–17 days p.i., the insulin release of the control islets was slightly reduced compared to that found at the earlier time points, suggesting a deterioration of the islet function due to long-term culture. The VD2921-infected islets did not release any insulin at all when stimulated with high glucose at this late time point (Table 3). The insulin and proinsulin contents were also slightly lower at this time point compared to day 7 but there was no difference between virus-infected and uninfected islets (Table 3).
TABLE 3. Insulin Release in Response to High Glucose, Insulin Content and Proinsulin Content in Human Pancreatic Islets Infected with CBV-4 VD2921 and in Uninfected Control Islets

<table>
<thead>
<tr>
<th>Human islets</th>
<th>Insulin release* mU/islet/24 hr Glucose conc.</th>
<th>Insulin content</th>
<th>Pro-insulin content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5.5 mM</td>
<td>16.5 mM</td>
<td>n (pg/islet)</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>30±30</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>370±110</td>
<td>2,600±1,600</td>
</tr>
<tr>
<td></td>
<td></td>
<td>370±110</td>
<td>4,260±1,600</td>
</tr>
<tr>
<td></td>
<td></td>
<td>370±110</td>
<td>70±55</td>
</tr>
<tr>
<td></td>
<td>VD2921-infected: 3–4 dpi</td>
<td>12</td>
<td>43±64</td>
</tr>
<tr>
<td></td>
<td>Controls: 3–4 dpi</td>
<td>12</td>
<td>330±70</td>
</tr>
<tr>
<td></td>
<td>VD2921-infected: 7–8 dpi</td>
<td>7</td>
<td>10±45</td>
</tr>
<tr>
<td></td>
<td>Controls: 7–8 dpi</td>
<td>7</td>
<td>360±150</td>
</tr>
<tr>
<td></td>
<td>VD2921-infected: 11–17 dpi</td>
<td>3</td>
<td>120±50</td>
</tr>
<tr>
<td></td>
<td>Controls-11–17 dpi</td>
<td>3</td>
<td>42±45</td>
</tr>
</tbody>
</table>

*Measurements of pro-insulin and insulin content were performed 6–7 days and 8–11 dpi, respectively.

Electron Microscopy of Virus-Infected Islets

Electron microscopy was performed to study virus-induced changes at the intracellular level and to locate the virus in the islet. This revealed crystalline inclusions with subunits corresponding to the size of picornavirus ~25 nm in β-cells of the islets infected with the VD2921 strain, but no other virus-induced morphological changes. In islets infected with the prototype strain of CBV-4 (JVB), crystalline inclusions were found in α-cells as well as β-cells. In some islet cells infected with the JVB strain, vesicular structures, assumed to be CBV-4-induced [95], were also found.

Genomic Sequence of the Persistent VD2921 Strain

The sequence of the VD2921 strain showed the highest amino acid (aa) identity with CBV-4 (the E2 strain) compared to other serotypes of CBV. The highest identity between the E2 strain and the VD2921 strain was found in the 5’NTR, VP1, VP2, VP3 and P2A regions (>90%, nt or aa) while the identity was higher to other serotypes of CBV in the other genome regions. Amino acid substitutions were found in all non-structural proteins of the VD2921 strain compared to the E2 strain.

The three-dimensional positions of eight of the seventeen substituted aa in VP1 of the VD2921 strain were predicted based on the structure of CBV-3 (Nancy strain) [152]. The substitutions (aa 142–144 and 158–162) were predicted to be located next to each other in the folded protein despite their separation in the aa chain and they were also placed next to the five-fold axis of symmetry in the capsid.

Two conserved aa motifs with homology to cellular proteins were found in VD2921. The immunogenic PALTAVETGHT-motif [153] with homology to heat shock protein 60 [73] was found in the VP1 region and the PEVKEK-motif with homology to GAD-65 [72] was found in the P2C region.
Study III

The Antiviral Effect of Pleconaril on Two CBV-4 Strains

Pleconaril inhibits the entry of most EV strains into cells by binding into and stabilising the viral capsid [131]. The aim of this study was to determine if a maximal pleconaril concentration (10 μM) could inhibit the replication of two different CBV-4 strains in isolated human islets. The two virus strains were chosen based on their differences in receptor usage in cell culture [86] and their different effects on human islets [70].

The results showed that the replication of both strains was affected by the treatment. The replication of the VD2921 strain was totally blocked by a single dose (Fig. 3B). The titres of the V89 4557 strain were reduced after pleconaril treatment but a pleconaril-resistant subpopulation was present and this phenotype continued to replicate (Fig. 3A). The selection of the resistant phenotype in the V89 4557 strain during pleconaril treatment was confirmed in a second islet infection where the replication of pleconaril-passaged virus was compared with the replication of virus from the original inoculum in the presence of pleconaril (Fig. 4).

No other characteristics were attributed to the pleconaril-resistant subpopulation of V89 4557. There was no change in the thermostability (at 46°C) of the pleconaril-passaged V89 4557 compared to the V89 4557 strain present in the original inoculum.

Figure 3. Virus replication in the presence or absence of 10 μM pleconaril in 50 cultured human islets infected with two different CBV-4 strains A) the V89 4557 strain (n=6) and B) the VD2921 strain (n=3). Virus titres are presented as means (log TCID₅₀/0.2ml). The two titres from day 3 (day 3-I and day 3-II) signify before and after the change of culture medium. The following experimental conditions are shown: islets inoculated with virus without pleconaril treatment (VD2921, n=3; V89 4557, n=6) (–×–); islets first inoculated with virus and then treated with pleconaril 30 minutes p.i. (VD2921, n=1; V89 4557, n=2) (–_hexagon–); islets first inoculated with virus and then treated with pleconaril 30 minutes p.i. and on day 3 after the change of culture medium (VD2921, n=2; V89 4557, n=4) (–_hexagram–); islets inoculated with a pleconaril-pretreated virus suspension (VD2921, n=3; V89 4557, n=6) (–▲–).
Figure 4. Pleconaril resistance test of the V89 4557 strain. Replication of pleconaril-passaged and pleconaril-unpassaged virus in the presence or absence of 10 μM pleconaril: pleconaril-passaged virus pretreated with pleconaril before inoculation, n=3 (–×–); unpassaged virus without addition of pleconaril, n=1 (– ▲ –); unpassaged virus pretreated with pleconaril before inoculation, n=1 (– ■ –). The TCID₅₀ titres (log TCID₅₀/0.2ml) are presented as means where applicable.

**The Effect of Pleconaril Treatment on Human Islets**

The effects of pleconaril and/or the solvent DMSO on human islets were assessed by light microscopy and measurements of glucose-stimulated insulin release.

Islets that had been cultured for six days in the presence of pleconaril together with 0.5% DMSO showed slightly more deterioration (degree of destruction on day six: 0–2.5+) than untreated islets (0–2+) although there were large variations between islets from different donors and between individual islets from the same donor. When DMSO was added alone (0.5%) to islet cultures, there was no apparent effect on islet appearance compared to untreated islets (n=3).

Glucose stimulations were performed in a dynamic perifusion system during two hours. Islets that had been exposed to 0.5% DMSO or to pleconaril together with 0.5% DMSO for two to four days were compared to untreated islets. In three of four perifusions, the insulin response of islets that had been treated with pleconaril together with DMSO was reduced compared to untreated controls and similar reductions were found after treatment with 0.5% DMSO alone (Fig. 5). One perifusion, however, did not show any reduction of the ability to release insulin after either of the treatments (Fig. 5: Donor 1 (day2)).
Figure 5: Insulin release during glucose perifusion of uninfected islets after two (donor 1), three (donors 1 and 2) or four days (donor 3) of culture untreated, or with addition of 10 μM pleconaril and/or 0.5% DMSO. Untreated control (– ▲ –); pleconaril+DMSO treatment (– △ –); DMSO treatment (– ● –).

Study IV

IP-10 Production in CBV-4-Infected Human Islets

The aim of this study was to measure the induction of the chemokine IP-10/CXCL10 in human islets and the secretion of IP-10 from these islets during CBV-4 infection. In addition, we wanted to compare this response between islets infected with three different strains of CBV-4 (E2, VD2921 and V89 4557).

The results showed a clear pattern of upregulation of IP-10 mRNA (Figure 6) and IP-10 protein (Fig. 7A) in islets after virus infection. All three virus strains were able to upregulate IP-10 mRNA on day 1 and day 2 p.i. and protein production was confirmed in islets infected with the VD2921 and V89 4557 strains. Islets infected with the VD2921 strain were studied until day 4 and IP-10 was upregulated in infected islets also on day 4 compared to uninfected islets (n=1). Upregulation of IP-10 protein in VD2921-infected islets on day 4–5 has been confirmed in additional experiments (n=2, G. Frisk unpublished observations). IP-10 secretion into the culture medium during the first 24 hours after infection was increased (p<0.05) from V89 4557-infected islets compared to that from uninfected controls (Fig.
Some IP-10 was secreted also from VD2921-infected islets but this did not reach statistical significance (n=3) (Fig. 7B).

Figure 6. Expression of IP-10 and β-actin mRNA after CBV-4 infection of human islets. Results are shown for islets from donors 2–6, infected with the VD2921 strain or the V89 4557 strain or uninfected. Islets were harvested for RT-PCR analysis before infection (day 0), 24 hours after infection (day 1) and 48 hours after infection (day 2).
Figure 7. A) The increase/decrease in IP-10 protein content in virus-infected and uninfected islets during the first 24 hours after infection. B) IP-10 secretion into the culture medium by virus-infected and uninfected islets during the first 24 hours after infection. Uninfected islets have been matched to virus-infected islets from the same islet donor and IP-10 secretion into the culture medium has been normalised to secretion per 50 islets. Data are presented as means±SD of three to five experiments as indicated. The asterisk denotes a statistically significant difference, p<0.05 (Wilcoxon Signed Ranks Test).
RINm5F Cells and RCC (Study II)

The aim of this study was to establish an alternative cell culture model for β-cells in which to study the effects of EV infection. First, the permissiveness of RINm5F cells to EV infection was investigated and second, the ability of the E2 strain of CBV-4 to induce NO production was studied in RINm5F cells grown as free-floating cell clusters (RCC).

The Ability of EV to Replicate in RINm5F Cells

Rat insulinoma-derived RINm5F cells were grown as monolayer cells and as free-floating cell clusters (RCC). Characterisation of the RCC showed that some cells were dividing in the cell clusters at the time of the virus inoculations (3–7 days after harvest) and that the insulin content was similar in the RINm5F monolayer and RCC cells.

Virus replication was studied after inoculation of both the monolayer and the RCC cultures with different serotypes of EV (CBV-1, -3, -4, CAV-7 and Echovirus-7). Despite the fact that none of these viruses replicated in the monolayer cultures of the RINm5F cells, they all replicated in the RCC with titre rises from $10^2$ to $10^3$ TCID$_{50}$/0.2ml.

Immunostaining to detect the CAR protein on the RINm5F monolayer and the RCC revealed expression of this CBV receptor molecule only on the RCC. Messenger transcripts (mRNA) encoding CAR were, however, detected by RT-PCR both in the RINm5F cells grown as monolayer cultures and in the RCC cells.

Cell Viability and NO Production in CBV-4-Infected RCC

Since all virus strains replicated to the same degree, the CBV-4 E2 strain was used for further studies of the effects of EV replication on RCC. The E2 strain replicated in the RCC for more than 10 days. Addition of rat IFN-γ to E2-infected cells reduced the virus replication if it was added 1 hour p.i. but not if it was added 24 hours p.i. (Table 4).

Table 4. Mean TCID$_{50}$ titres of the CBV-4 E2 strain in RINm5F cells cultured as free-floating RCC, with and without addition of rat IFN-γ

<table>
<thead>
<tr>
<th>Virus</th>
<th>n</th>
<th>day 0</th>
<th>day 1</th>
<th>day 3</th>
<th>day 1-3</th>
<th>day 4-7</th>
<th>day 8-10</th>
<th>day &gt;10</th>
</tr>
</thead>
<tbody>
<tr>
<td>E2</td>
<td>7</td>
<td>$10^{1.8}$</td>
<td>$10^{2.1}$</td>
<td>$10^{1.8}$</td>
<td>$10^{6.0}$</td>
<td>$10^{5.6}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2+ rat IFN-γ 1hpi</td>
<td>5</td>
<td>$10^{1.9}$</td>
<td>$10^{2.75}$</td>
<td>$10^{0.9}$</td>
<td>$10^{0.5}$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E2+ rat IFN-γ 24hpi</td>
<td>7</td>
<td>$10^{2.3}$</td>
<td>$10^{3.4}$</td>
<td>$10^{5.5}$</td>
<td>$10^{4.9}$</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

hpi = hours post infection
Cell viability in the free-floating RCC after CBV-4 infection was assessed by light microscopy and trypan blue staining. The appearance of the RCC suggested a very low degree of destruction of the infected RCC, although some clusters did show a slight loss of cells from around the edges leading to the accumulation of cells in the surrounding culture medium. In the cases where virus replication was blocked by the addition of IFN-γ, the infected RCC did not differ in appearance to uninfected RCC. Trypan blue staining after trypsinisation of the infected RCC did not reveal a statistically significant difference in viability between E2-infected and uninfected RCC on day 3 or day 7 p.i. despite a slight reduction in the number of viable cells in the infected RCC on day 7 (Table 5). With increasing time in culture, uninfected as well as infected RCC gained a denser appearance in the centres. Light microscopy of sections of these dense RCC (cultured for 14 days after harvest) showed necrosis in the centres of the clusters and also pyknotic cell nuclei in some RCC.

Table 5. Viability of cells in free-floating RCC assessed by trypan blue staining

<table>
<thead>
<tr>
<th></th>
<th>Day 3</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Uninfected RCC</td>
<td>87±5% (n=5)</td>
<td>59±11% (n=5)</td>
</tr>
<tr>
<td>CVB-4-E2-infected RCC</td>
<td>86±6% (n=4)</td>
<td>35±10% (n=5)</td>
</tr>
<tr>
<td>Untreated RCC</td>
<td>86±4% (n=8)</td>
<td>42±5% (n=8)</td>
</tr>
<tr>
<td>Poly(IC) 100 µg/ml + IFN-γ 100 U/ml</td>
<td>61±3% (n=6)*</td>
<td>37±11% (n=6)</td>
</tr>
<tr>
<td>Poly(IC) 400 µg/ml + IFN-γ 500 U/ml</td>
<td>61±8% (n=4)*</td>
<td>32±10% (n=4)</td>
</tr>
</tbody>
</table>

Data are presented as means±SEM
*Differs from untreated control, p<0.05

The NO production of the RCC was measured as nitrite accumulation in the culture medium. Virus infection (CBV-4 E2) alone did not induce NO production in the RCC, nor was there any NO production induced when IFN-γ was added to virus-infected islet cultures (Fig. 8). In contrast, addition of artificial dsRNA, poly(IC), and IFN-γ induced NO in a dose-dependent manner (Fig. 9). Viability was also reduced in RCC treated with poly(IC) in combination with IFN-γ (Table 5). Neither poly(IC), nor IFN-γ induced NO production when added separately to the RCC (data not shown).
Figure 8. Nitrite concentration, means±SD, in culture medium from uninfected RCC (n=4), RCC infected with CVB-4-E2 without addition of IFN-γ (n=4) or with IFN-γ added 1h (n=4) or 24 h (n=2) post infection. RCC treated with IFN-γ alone (n=4) and culture medium without cells (n=2) are also shown.

Figure 9. Nitrite concentration in culture medium, means±SD, from RCC treated with Poly-IC (100 µg/ml) together with IFN-γ (100 U/ml) or Poly-IC (400 µg/ml) together with IFN-γ (500 U/ml), n=3. Untreated control RCC and culture medium without cells are also shown.
Discussion

In order to prove or disprove a causal relationship between EV infections and T1D, potential mechanisms of disease induction must be examined. This thesis has focused on the hypothesis that β-cell destruction in T1D involves an EV infection of the islets. The studies have mainly investigated the effects of EV infection on the β-cell and the ability of an EV infection of the islets to induce different mediators that might promote the destruction of the β-cells. The availability of human pancreatic islets from organ donors has provided a unique tissue culture model for the study of virus interactions with human host cells. In addition a new β-cell culture model of rat insulinoma cells growing in clusters was established for EV infections.

EV Infection of the RINm5F Cell Line as a Model for EV Infections of β-Cells (II)

The RINm5F cell line has frequently been used in studies of β-cell death mechanisms [138-140, 154] and was therefore evaluated as an alternative model in which to study EV infections in β-cells. To our surprise, we found that EV did not replicate in the RINm5F monolayer cells, while they replicated well in the RINm5F cells that were growing as free-floating cell clusters (RCC). When the expression of the CBV receptor CAR was examined by immunostaining, it was found that the CAR protein was only detectable on the RCC. Meanwhile, CAR mRNA was expressed in both RINm5F monolayer cells and RCC. The reason for this discrepancy is not known, but the difference in CAR protein expression might explain why the CBV strains could only replicate in the RCC. The acquired permissiveness of the RCC to the other EV, which are not known to use CAR for entry, suggests that perhaps other cell surface proteins or other unidentified intracellular factors necessary for virus replication might also change their expression when the cells start to grow as clusters. If insulin-producing cells can change their permissiveness to EV in vivo, this finding may represent an important factor in the susceptibility of β-cells to EV infection and to T1D development. Extrapolations from this rat insulinoma cell line to human β-cells, must however be done with some caution. Rodent β-cells and continuous cell lines naturally differ somewhat from human β-cells due to a lower degree of differentiation and also due to species differences.
Replication of the CBV-4 strains JVB and E2 in the RCC caused no or only slight destruction of the cell clusters. This differs from when human islets are infected with these two strains which usually results in clear CPE, manifested as a gradual destruction of the islets [70]. The reason for the absence of CPE in RIN cells may be that unlike the β-cells of isolated islets, the RIN cells continue to divide in the RIN cell clusters, as visualised by 3H-thymidine incorporation, and this might compensate for the loss of cells due to virus-induced lysis. Another explanation for the lack of CPE could be that not all cells of the RCC are susceptible to infection, due to a variability in CAR expression. Immunostaining for CAR did not stain all cells of the RCC, indicating that this might be the case. The extent of CAR expression on human islet cells has not been investigated, but they are permissive to CBV and blocking experiments using a monoclonal antibody directed against CAR can reduce the attachment [94] and replication [69] of CBV-4 in islets, indicating that CAR is expressed also on human islets.

In conclusion, the free-floating RCC expressed the CBV receptor CAR and were permissive to infections with different serotypes of EV including the CBV-4 strains E2 and JVB which can also infect human islets. The RCC were thus considered to be a useful model for further studies of EV–β-cell interactions, especially for studies requiring large numbers of cells.

The Effects of EV on Islet Cells

It has previously been shown that the outcome of an EV Infection of isolated human islets is mainly dependent on the virus strain [70, 126] and can range from complete islet destruction to little or no effect on islet morphology. Therefore, several different EV strains with both islet destructive and non-islet-destructive properties were studied.

β-Cell Function is Largely Preserved in Persistently EV-Infected Islets (I)

The CBV-4 VD2921 strain replicated in human islets for 15 days without inducing CPE and without affecting the proinsulin or insulin content of the islets. The ability to secrete insulin in response to stimulation with high glucose was maintained in virus-infected islets at least up to 7 days p.i.. Moreover there was no effect of the VD2921 strain on the intracellular morphology of the islet cells despite the detection of virus-like particles in the β-cells. These findings confirmed the previous report that this strain could replicate without causing degeneration of human islets under conditions that resulted in islet cell lysis with other CBV-4 strains [70]. Non-lytic replication in human islets with individual strains has also been described for other EV serotypes: CAV-9, Echovirus-1, -9 and -30 [71, 126].

Receptor usage has been suggested to be an important determinant of the ability of EV to persist in cells and a few amino acid changes in VP1 are sufficient to change the phenotype of a virus from non-lytic to lytic [155].
The genomic sequence of the VD2921 strain revealed seventeen amino acid differences in the VP1 capsid protein compared to the lytic E2 strain. The predicted location of eight of these was on the surface of the capsid close to the receptor-binding canyon [156], which suggests that they might affect interactions with host cell receptors. Antibody-mediated blocking of receptors on continuous cell lines also suggests differences in receptor usage between VD2921 and the lytic E2 and JVB strains [86]. Thus, different receptor usage by this strain might be one of many possible reasons for the persistence in islet cells.

The insulin and proinsulin content of the islets was unchanged by the VD2921 infection, which indicates either that not all β-cells were infected or that host cell protein translation was not shut off during the infection with the VD2921 strain. The membrane rearrangements that were seen in the JVB-infected but not in the VD2921-infected β-cells suggest that VD2921 replication has less deleterious host cell effects than the more lytic strains. Sequence differences were also found in the non-structural proteins that mediate host cell translational shut off (protein 2A) [99] and membrane rearrangements (proteins 2BC and 3A) [157] when compared to the lytic E2 strain. This could indicate a difference in function of these proteins that might also be involved in the persistence of the VD2921 strain, although the precise effects of these mutations on protein activities have not been analysed.

The properties that enable a virus to persist in islets in vitro might also facilitate persistence in vivo. In the long term, persistent virus replication in β-cells might be damaging to the β-cell as suggested by the reduction of insulin release in VD2921-infected islets at 11–17 days p.i. in this study.

Nitric Oxide Production is not Induced in Insulin-Producing Cells by the Lytic Virus Strain CBV-4 E2 with or without IFN-γ (II)

The CBV-4 E2 strain which can cause islet destruction in isolated human islets [70] was used to study NO production after virus infection and IFN-γ treatment in insulin-producing (RINm5F) RCC cells. The virus replicated in the RCC up to 10 days p.i. but did not induce NO production in the cells even after the addition of IFN-γ. Furthermore, the slight virus-induced effects on the appearance of the RCC were not aggravated by the addition of IFN-γ, which might have been expected if toxic amounts of NO had been induced. When IFN-γ was added 1 hour p.i. it reduced virus replication also without inducing NO production. Altogether the data suggest that IFN-γ does not exert its antiviral effects in insulin-producing cells via induction of NO and that NO production is not likely to be a major cause of β-cell damage during EV infection even in the presence of IFN-γ.

NO production in macrophages in vivo can be triggered by the presence of viral products such as dsRNA and cytokines and is believed to be mostly tissue-protective due to the ability of NO to reduce virus replication [158]. In
some cases, however, NO can mediate inflammatory damage to cells and this has been proposed as a mechanism by which viruses might induce damage to $\beta$-cells [123]. The synthetic dsRNA molecule, poly(IC), often used to mimic a replicating virus, cannot alone stimulate upregulation of the enzyme iNOS which catalyses formation of NO. However, when added together with IFN-\(\gamma\), poly(IC) induces production of NO in islet resident macrophages [118] and in rodent $\beta$-cells [123] in amounts that are toxic to rodent $\beta$-cells. NO production has not been detected in association with EV replication in previous studies in insulin-producing MIN-6 cells [159] or in isolated human islets [71], which is in agreement with our findings in CBV-4-infected RCC. The lack of NO production during EV infection together with IFN-\(\gamma\) in $\beta$-cells was however a new and unexpected finding considering the data from poly(IC) and IFN-\(\gamma\).

It has been suggested that this cell type (RINm5F cells) might be unable to respond to dsRNA and IFN-\(\gamma\) treatment [118, 160], although they can upregulate iNOS after IL-1 exposure [118]. The present study, however, showed a clear increase in nitrite, indicative of NO production accompanied by morphological signs of degeneration and reduced cell viability, in RCC cultures treated with poly(IC) together with IFN-\(\gamma\). The changed responsiveness to poly(IC)+IFN-\(\gamma\) treatment of the RCC compared to the RINm5F cells used in previous studies might be related to the altered growth pattern of these cells, which also resulted in permissiveness to EV infection. These changes may include modified expression of cell surface proteins or intracellular signalling molecules necessary for activation of iNOS transcription. Since the RCC used in this study were able to mount an NO response to dsRNA+IFN-\(\gamma\), another explanation must be sought for why IFN-\(\gamma\) treatment during CBV-4 infection did not stimulate a similar response. An EV infection introduces more components into the cell than dsRNA, including several viral proteases that could potentially block necessary signalling pathways. Viral proteases may inactivate transcription factors e.g. NF-\(\kappa\)B [113], which is important for iNOS transcription [118-120]. Host cell translation may be shut off by virus protein 2A [99], preventing the formation of iNOS protein. Alternatively, EV dsRNA, which is formed as a replicative intermediate, may not be accessible to the cell in the same way as poly(IC), perhaps due to RNA-binding proteins or due to the arrangement of the viral replication complex [161].

In conclusion, NO was found not to be induced during CBV-4 infection and IFN-\(\gamma\) treatment of these insulin-producing cells. This showed that poly(IC) does not mimic an EV infection in this respect and suggests that $\beta$-cell production of NO is not a major mediator of damage during virus infections of islets even under conditions when IFN-\(\gamma\) is expressed. It cannot be excluded that other cell types in the islets, notably macrophages, might be stimulated to produce NO by viral products and IFN-\(\gamma\) [162, 163] in vivo,
although the ability of NO to damage human β-cells has been questioned [17, 18].

**IP-10 is Induced in Islet Cells by Lytic and Non-Lytic EV Strains (IV)**

Isolated human islets upregulated IP-10 mRNA expression during infection with the lytic CBV-4 strains E2 and V89 4557 and the non-lytic CBV-4 strain VD2921. More importantly, IP-10 protein was also produced and secreted from these cells, which shows that this host cell response is not blocked even by lytic virus strains. All three virus strains induced IP-10 on days 1 and 2 p.i. and no virus strain differences were seen in the degree of response. The late time point after 4 days of infection was only studied with the non-lytic VD2921 strain and showed that IP-10 could be increased also on day 4. This chemokine is involved in the recruitment of activated Th1 type T-lymphocytes to tissues during inflammation [164, 165] and islet expression of IP-10 in vivo seems to play an important role in the early stages of insulitis in mouse models of T1D [166-168].

It has previously been shown in other cell types that picornavirus infection can induce IP-10 expression and secretion [169, 170], which supports our finding in human islets. In addition, a recent microarray study found that IP-10 mRNA was upregulated in human islets infected with CBV-5 [128] which also indicates that this response is probably not limited to infections with certain serotypes of EV.

The fact that both lytic and non-lytic EV strains induced production of IP-10 protein in human islets suggests that IP-10 induction in islets is unaffected by EV-induced translational shut off in the host cell, which is believed to occur during most lytic infections [171]. For example, IP-10 might be translated by a cap-independent mechanism as some cellular proteins are [100]. Different mechanisms might also be responsible for the induction of IP-10 during lytic compared to non-lytic EV infections. Studies of the mechanisms in other cell types have shown that transcription factor NF-κB [169, 170] and the p38 MAP kinase [169] are both important. They have also revealed that exposure of cells to dsRNA (poly(IC)) can activate NF-κB and p38 MAP kinase [172] and induce IP-10 secretion [160, 170]. In macrophages [121, 163] and in astrocytes [173] it has also been shown that NF-κB and p38 MAP kinase are activated within 30–60 minutes of exposure to picornavirus even in the absence of viral RNA [163], suggesting that signals leading to IP-10 transcription might be triggered already by virus capsid interactions with cell surface receptors. This would allow IP-10 expression to begin before virally encoded proteases could inactivate NF-κB [113] or cellular translation [171]. In agreement with this, expression of chemokines, including IP-10, can be detected within hours of infection, suggesting that at least some of the chemokine production is an early response of the picornavirus-infected cell [169]. In this study, the duration of the IP-10 secretion from human islets was also studied. The increased amounts of IP-10 that
were detected in virus-infected islets and in the culture medium until day 4 in the present study may reflect the gradual exposure of uninfected cells to virus during an islet infection.

To conclude, infection of human islets with lytic or non-lytic EV strains resulted in IP-10 secretion. A similar viral induction of IP-10 in islets in vivo might lead to recruitment of T-cells to the islets and trigger insulitis. This could be a common mechanism by which infections with \( \beta \)-cell tropic viruses enhance immune-mediated destruction of \( \beta \)-cells. The ability of the non-lytic VD2921 strain to induce IP-10 in islet cells shows that persistent virus infection might also increase the risk of immune-mediated destruction of \( \beta \)-cells and perhaps viral persistence could lead to a prolonged stimulation of the immune system.

Antiviral Treatment of EV Infections in Islets (III)

**Pleconaril Treatment Reduces the Replication of VD2921 and V89 4557 in Islet Cells (III)**

Treatment with a high concentration of the anti-enterovirus [133] and anti-rhinovirus [134] compound pleconaril reduced the replication of both the lytic (V89 4557) and non-lytic (VD2921) CBV-4 strains in human islets. However, while the replication of the VD2921 strain was completely blocked, a subpopulation of the V89 4557 strain was resistant to the treatment and continued to replicate in the islets despite pleconaril treatment. This clearly demonstrated differences in susceptibility between these two virus strains to pleconaril but also showed that antiviral treatment can successfully block the replication of some EV in islet cells.

At the concentration used, pleconaril (using 0.5% DMSO as a solvent) had a slight effect on the appearance of uninfected islets. Due to the shortage of this kind of cells a high pleconaril concentration was chosen to see if this would block virus replication. This concentration had earlier been shown not to be toxic to cell cultures [133].

Pleconaril (dissolved in 0.5% DMSO) reduced the glucose-stimulated insulin response of the islets in three of four glucose perifusions. Similar reductions were found in islets from the same donors treated with 0.5% DMSO without pleconaril indicating that the effect might have been due to DMSO. When pleconaril is used to treat human subjects, DMSO is not used as a solvent [174]. Therefore effects of DMSO on islet function would not be a problem if pleconaril were to be used in clinical trials to treat EV infection in T1D patients.

Pleconaril resistance has been attributed to mutations affecting the drug-binding pocket located in protein VP1 in the virus capsid [175]. Such mutations can also affect the stability of the capsid and change properties such as the sensitivity to heat [175] due to the function of the pocket for virion stability [131]. There was no increase in heat sensitivity in the pleconaril-
resistant V89 4557 variant selected during passage in islets compared to the virus in the original inoculum, suggesting that the resistant variant did not differ in this respect to the susceptible virus. A difference in receptor usage between the VD2921 and V89 4557 strains on various cell types [86, 146] indicates that their VP1 sequences might differ and this might affect also the drug binding pocket.

In summary, EV replication in human islets can be reduced by antiviral treatment in vitro. All β-cell tropic strains are not sensitive to pleconaril but some are completely blocked. This suggests that antiviral treatment of EV infections in islets to prevent β-cell loss in T1D might feasible if EV are shown to be true causal agents. However, until the properties of the EV strains involved in T1D have been defined, other antiviral strategies should be examined to find a treatment also for pleconaril-resistant EV strains.

### The Role of EV Infections of β-Cells in T1D

The results presented in this thesis provide new insight into the effects that EV infections have on the β-cell and on other islet cells. Such knowledge can be used to evaluate the role of this virus in T1D. In addition, the possibility to treat such infections with an antiviral compound was examined in a pilot study. Based on the presented findings, the genetics of the virus strain is a significant determinant of the degree of damage it causes to the β-cell, while the ability to stimulate IP-10 production and secretion from islets seems to be a more general property of CBV-4 strains. The ability to induce NO in insulin-producing cells does not seem to be an inherent property of lytic strains of EV. The importance of the host for EV replication was demonstrated in the RINm5F cell line, in a study that showed that changes in insulin-producing cells related to the growth pattern can result in permissiveness to EV. The pleconaril study revealed that a single antiviral strategy might not suffice to combat all infections with β-cell tropic EV strains although some of these EV infections were clearly treatable in human islets.

One of the major strengths of these studies lies in the use of human islet tissue, which mimics as closely as is currently possible the situation of an EV infection in islets in vivo. Of course, due to the isolation procedure and the in vitro culture conditions, it cannot be excluded that the islet cells do not react entirely as they would in a similar situation in vivo. Thus, these studies do not provide a final answer to the question of whether EV infections can cause T1D. The findings nonetheless show evidence that supports several current hypotheses of the disease process and enable a discussion on the potential of EV to induce T1D and the mechanisms that might be involved in vivo.

One can speculate that a virus strain which does not immediately disturb β-cell function may be able to persist in the islet cells for a long time. In vivo, this might lead to long term β-cell damage caused by the virus itself or
by an immune response aimed at clearing the persisting virus. Destructive immune cells might be recruited by IP-10 and possibly other chemokines secreted from the infected cells induced by the persisting virus. A lytic virus could, as has been described earlier [70, 71], destroy β-cells directly, probably by NO-independent mechanisms. The concurrent induction of IP-10 secretion might also trigger insulitis during a lytic infection. Whether any of these mechanisms operate in vivo and lead to T1D remains to be determined.

To conclude, there is still reason to believe that EV infections of the islets could contribute to T1D development.
Conclusions

The main conclusions drawn from the results are as follows:

- RCC are susceptible to many serotypes of EV and represent a new rodent β-cell model in which to study effects of EV infection or factors determining permissiveness to EV infection.

- Some EV can replicate in β-cells without affecting insulin production or secretion and this may increase the EV strain’s ability to persist.

- EV infection, unlike synthetic dsRNA, does not induce NO production in RCC in the presence of IFN-γ, which suggests that poly(IC) is not a good model for EV infection in β-cells.

- The chemokine IP-10 is induced in and secreted from human islets infected by both lytic and non-lytic EV strains and may represent the link between virus infection and insulitis.

- Antiviral treatment reduced the viral replication of two different CBV-4 strains in human islets.

In summary, the studies presented in this thesis have increased our understanding of the effects of EV infection on human and rat β-cells. Whether these effects lead to T1D in vivo remains to be determined.
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