Studies of the Effect of Enterovirus Infection on Pancreatic Islet Cells

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Abstract


Enterovirus (EV) infections have been associated with the pathogenesis of Type 1 Diabetes (T1D). However, the pathway(s) by which EV may induce or accelerate diabetes is not well understood. The purpose of this thesis was to obtain new information on the mechanism by which EV infections, with different strains of EV, could cause damage to the insulin-producing \( \beta \)-cells in isolated human islets and in a rat insulin-producing cell line (RINm5F).

Infection with EV strains isolated from T1D patients revealed replication/cell destruction in human islets and EV-like particles in the cytoplasm of the \( \beta \)-cell and infection with the isolates affected the release of insulin in response to glucose stimulation as early as three days post infection, before any decrease in cell viability was observed. A decrease in the induction/secretion of the chemokine RANTES in human islets during EV infection was also detected. When islets were cultured with nicotinamide (NA) the secretion of RANTES was increased irrespectively if the islets were infected or not. In addition, the degree of virus-induced cytolysis of human islets was reduced by NA, suggesting an antiviral effect of NA. Infection with EV strains revealed permissiveness to islet-derived cells.

All EV strains used for infection were able to replicate in the RIN cell clusters (RCC) but not in the RIN cells that were cultured as a monolayer. This might be due to the differences in expression of the Coxsackie-adenovirus receptor (CAR), which only could be detected on the RCC. Infection of RCC with a CBV-4 strain did not affect cell viability and did not induce nitric oxide (NO) production alone or with the addition of IFN-\( \gamma \). This was in contrast to the results obtained with synthetic dsRNA, poly(IC), which induced NO, suggesting that synthetic dsRNA does not mimic enteroviral intermediate dsRNA.

During analyses performed with the samples from a family where the mother and one son where diagnosed with T1D on the same day, the results showed that the whole family had a proven EV infection at the time diagnosis.

To conclude, the ability of EV strains to replicate in RIN cells is dependent on the growth pattern of the cells and this may be due to the upregulation and/or changed expression pattern of CAR in these cells. In the RIN cells, contrary to artificial dsRNA, viral dsRNA does not induce NO. The isolated EV virus strains used were able to infect and affect human pancreatic islets in vitro. The chemokine RANTES is reduced during an EV infection of human pancreatic islets and NA causes upregulation of RANTES in infected and uninfected islets.

Keywords: type 1 diabetes, Human pancreatic islets, \( \beta \)-cells, chemokines, enterovirus

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To 

Ibrahim and Zeinab
List of Publications

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

I  dsRNA formed as an intermediate during Coxsackievirus infection does not induce NO production in a beta-cell line with or without addition of IFN-gamma.

II Simultaneous type 1 diabetes onset in mother and son coincident with an enteroviral infection.

III Effects on isolated human pancreatic islet cells after infection with strains of enterovirus isolated at clinical presentation of type 1 diabetes.

IV RANTES/CCL5 secretion during enterovirus infection of isolated human pancreatic islets and human islets derived cells.
   Elshebani, A., Álin, E., Tuvemo, T., Korsgren, O., Frisk G. (manuscript)
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## Abbreviations

<table>
<thead>
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<th>Description</th>
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</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>analysis of variance</td>
</tr>
<tr>
<td>ATCC</td>
<td>American Type Culture Collection</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CAR</td>
<td>Coxsackie-adenovirus receptor</td>
</tr>
<tr>
<td>CAV</td>
<td>Coxsackie A virus</td>
</tr>
<tr>
<td>CBV</td>
<td>Coxsackie B virus</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary cell line</td>
</tr>
<tr>
<td>CPE</td>
<td>cytopathic effect</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
</tr>
<tr>
<td>DAF</td>
<td>decay accelerating factor</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>dsRNA</td>
<td>double-stranded ribonucleic acid</td>
</tr>
<tr>
<td>EIA</td>
<td>enzyme immunoassay</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>EMEM</td>
<td>Eagle’s Minimum Essential Medium</td>
</tr>
<tr>
<td>EV</td>
<td>enterovirus</td>
</tr>
<tr>
<td>GAD</td>
<td>glutamic acid decarboxylase</td>
</tr>
<tr>
<td>GLIMA</td>
<td>glycated islet cell membrane-associated</td>
</tr>
<tr>
<td>GMK</td>
<td>green monkey kidney cell line</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HeLa</td>
<td>human tumor-derived cell line</td>
</tr>
<tr>
<td>HLA</td>
<td>human leucocyte antigen</td>
</tr>
<tr>
<td>IA-2</td>
<td>insulinoma-associated protein 2</td>
</tr>
<tr>
<td>ICA</td>
<td>islet cell antibody</td>
</tr>
<tr>
<td>ICAM</td>
<td>intercellular adhesion molecule</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IRES</td>
<td>internal ribosome entry site</td>
</tr>
<tr>
<td>MAbs</td>
<td>monoclonal antibodies</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility complex</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MyD88</td>
<td>myeloid differentiation factor 88</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-κB</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOD</td>
<td>non-obese diabetic</td>
</tr>
<tr>
<td>NT</td>
<td>neutralisation</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffer saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>para-formaldehyde</td>
</tr>
<tr>
<td>poly(IC)</td>
<td>poly-inosinic-poly-cytidylic acid</td>
</tr>
<tr>
<td>PV</td>
<td>poliovirus</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated on activation, Normal T-cell Expressed</td>
</tr>
<tr>
<td>RCC</td>
<td>RINm5F cell clusters</td>
</tr>
<tr>
<td>RINm5F</td>
<td>rat insulinoma m5F cells</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RD</td>
<td>Rhabdomyosarcoma cell line</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>SEM</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>ssRNA</td>
<td>single-stranded RNA</td>
</tr>
<tr>
<td>T1,2</td>
<td>Tuwemo 1,2</td>
</tr>
<tr>
<td>T1D</td>
<td>type 1 diabetes</td>
</tr>
<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>tissue culture infectious dose-50</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
<tr>
<td>VP</td>
<td>viral protein</td>
</tr>
</tbody>
</table>
Background

Human Enteroviruses

Enteroviruses (EV) are small non-enveloped viruses, belonging to the Picornaviridae family. They have been divided into five species, Poliovirus (PV) and Human enterovirus (HEV) A-D according to their genetic relationship [1], but recently it has been suggest that polioviruses should be reclassified as members of HEV-C [2], which has been approved by the International Committee on Taxonomy of Virus executive committee [3] (Table 1).

Table 1. Classification of EV.

<table>
<thead>
<tr>
<th>Species</th>
<th>Subgroup and serotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human Enterovirus A (HEV-A)</td>
<td>CAV2-8, 10, 12, 14, 16; EV71, EV76, EV89, EV91</td>
</tr>
<tr>
<td>Human Enterovirus B (HEV-B)</td>
<td>CAV9; CBV1-6; E1-7, 9, 11-21, 24-27, 29-33; EV69, EV73-75, EV77-88, EV100-101</td>
</tr>
<tr>
<td>Human Enterovirus C (HEV-C)</td>
<td>CAV1, 11, 13, 17, 19-22, 24, PV1-3</td>
</tr>
<tr>
<td>Human Enterovirus D (HEV-D)</td>
<td>EV68, 70</td>
</tr>
</tbody>
</table>

The Enterovirus Genome

The EV genome consists of a single-stranded RNA of positive polarity and about 7500 nucleotides in length, with a small virus-encoded protein VPg attached to the 5’ end of the molecule. At both the 5’ end and 3’ end of the genome, there is an un-translated region (UTR). The 5’ UTR makes up approximately 10% of the genome and is important in translation, virulence, and directing ribosomes into the internal ribosome entry site (IRES) [4, 5].
The 5’ UTR is followed by an open reading-frame encoding a single polyprotein. The 3’ UTR is shorter and followed by a poly-A tail, and the 3’ UTR is necessary for the negative strand synthesis during replication [6]. The genomic RNA acts as a template for the production of viral proteins needed in translation and replication.

The Enterovirus Life Cycle

In order to initiate an infection, the virus must first bind to its specific receptor molecule on the surface of the target cell. The expression of specific receptors is an important determinant of virus host range and tissue tropism. Attachment to these molecules is specific, and during evolution, viruses have adapted to use a variety of receptors. Several receptors have been demonstrated for EV such as the coxsackie-adenovirus receptor (CAR) [7], decay accelerating factor (DAF) [8, 9], intercellular adhesion molecule 1 (ICAM-1) [8, 10], heparan sulphate [11] and some integrin receptors [12-15]. Once the virus is attached to a cell surface receptor on a susceptible cell, the genomic RNA is translocated across the cell membrane into the host’s cytoplasm. The genomic RNA might act directly as mRNA, once it is inside the cell [16]. The complete viral protein-coding region encodes a single polyprotein, which proteolytically cleaved into precursor proteins P1, P2 and P3. P1 is further cleaved into three capsid (structural) proteins VP0, VP3 and VP1 [17], of which VP0 is cleaved to VP2 and VP4 during capsid assembly. P2 and P3 are also further cleaved into seven non-structural proteins, which have functions in virus replication and encapsidation of viral RNA and in protein processing. Proteins 2A, 3C and 3CD are proteases, 3D is the RNA-dependent RNA polymerase, 2C is apparently a helicase, and has a function in encapsidation of RNA [18]. The same genomic RNA is also used as a template for the synthesis of a negative strand RNA molecules by the virus-encoded polymerase 3D. The negative strand RNA copy is then used for synthesising multiple copies of positive strand RNA. The new positive strand RNA molecules are used in translation of viral proteins, and are encapsidated into the assembling virions.

EV Infection

EV infection of the cell can lead to different outcomes, that is, EV can replicate and induce morphological changes in the host cell, known as cytopathic
effect (CPE), which include cell rounding and cell detachment leading to cell death [16]. The virus can also replicate with a low rate without killing the cell, a persistent infection [19]; Frisk, 2001 #120}. When the virus crosses the cell membrane and gets access to the cellular synthetic machinery in the cytoplasm, virus can take over the host cell translation machinery and cause inhibition of the host cell protein synthesis (shut-off) [20, 21], which is believed to be mediated by viral protein 2A and protease 3C [21]. Cellular transcription can also be inhibited as shown with poliovirus protease 3C [22-24], which then alters the host cell gene expression pattern during the virus infection. EV can also interfere with intracellular protein trafficking as shown with the coxsackievirus 3A protein that can block the intracellular transport of vesicles from the ER to the Golgi [25, 26]. All these processes in host cells during the virus infection can affect the pathogenesis in the target tissue.

The host cells defend themselves and mount an appropriate antiviral response. For example, the dsRNA formed during EV replication in the host cytoplasm is an important cellular alerting signal [27, 28]. The dsRNA can be recognized by the cells through different pathways such as the dsRNA-dependent protein kinase (PKR) and the 2’, 5’ oligoadenylate synthetase [29]. In addition, the Toll-like receptor-3 (TLR-3) has also been shown to recognize and bind to dsRNA [30]. Transcription factor, nuclear factor-κB (NF-κB) and the mitogen-activated protein kinases (MAPKs) are then activated, via a myeloid differentiation factor 88 (MyD88)-dependent and/or independent pathway[28, 30, 31]. Activation of TLR3 via dsRNA was shown to lead to inflammatory cytokine / chemokine release [32] and production of type I interferon (IFNs) [28].

Clinical Manifestation of EV Infections

EV infections are very common in humans and close human contact is required for the spread of the virus. EV are transmitted by the fecal-oral route in most cases, but transmission via either the upper respiratory tract or the conjunctiva of the eye [33], and from mother to the fetus via the placenta [34] are possible. Because of their stability at very low pH, they can survive passages through the stomach to infect the intestinal tract, where the infection may remain subclinical or result in mild symptoms. In some cases, the
virus spreads through the lymphatics into the circulation causing viremia and may spread to secondary sites of replication in specific tissues and organs [35]. EV infections are most common during late Summer and Autumn. A great majority of EV infections are asymptomatic, but they can also cause a variety of diseases from mild upper respiratory infection to poliomyelitis and meningitis/encephalitis. EV have also been indicated in chronic inflammatory diseases, such as chronic myocarditis [36, 37], type 1 diabetes [38-47] and Sjögren's syndrome [48-50].

Diagnosis

From the asymptomatic infection to the severe diseases, diagnosis has earlier depended mostly on virus isolation. Isolation can be applied to all types of clinical specimens, e.g. stool, rectal swabs, throat swabs, cerebrospinal fluid (CSF), and blood specimens. After isolation in cell culture, the isolate is serotyped by neutralization (NT) with antiserum pools. However, some factors may impair the interpretation of the NT tests, unstable genomes enable neutralizing escape mutants to occur at high rates, and the isolate may be a mixture of viral types [35] in addition it could be a previously undescribed serotype [51]. In addition to isolation, several enzyme immunoassays (EIA) for the detection of enteroviral IgM, IgG, IgA antibodies can be used for EV diagnosis [52-56]. The introduction of polymerase chain reaction (PCR) methods has markedly improved the speed and sensitivity of clinical EV detection. Methods based on amplification of enteroviral genomic sequences by reverse transcriptase RT-PCR are useful diagnostic tools. These assays can detect most or all EVs when the primer sequences are located in highly conserved genomic regions, which have been identified in the 5' non-coding region of the EV genome [57-60].

Type 1 Diabetes (T1D)

Type 1 diabetes (T1D) is a chronic disease known as insulin-dependent diabetes mellitus, meaning that insulin therapy is required from the onset of the disease [61]. The cause of this condition is the progressive destruction of the insulin-producing β-cells in the islets of Langerhans the pancreas. T1D is also called juvenile diabetes, as the onset is usually before the age of 35 [61]. The incidence of T1D is globally increasing by 3.0% per year [62], and the incidence varies considerably between different continents and countries.
Pathogenesis

The β-cell loss in T1D is considered to result from a multifactorial process, involving host genes, autoimmune responses, and cytokines as well as environmental factors [65-69].

Genetic predisposition has traditionally been considered to originate from the class II major histocompatibility complex (MHC) genes on chromosome 6p21, and is associated with HLA – DR and DQ [70, 71]. These genes are expressed on the surface of antigen-presenting cells, and present antigenic peptides to CD4 T-lymphocytes [72]. Approximately 30% of T1D patients are heterozygous for the high risk HLA-DR3/4 or HLA-DQ2/DQ8. Some also lack the protective HLA-DR2 or HLA-DQ6 which increases T1D susceptibility [73].

Autoimmunity in the case of T1D is a type of self-allergy that causes the T-lymphocytes (or T cells) of the immune system to attack the pancreatic islets as if they were a foreign invader. Research on the autoimmune response toward islet cell antigens has shown that it can be demonstrated in pre-diabetic individuals often several years before clinical presentation of diabetes [74]. Islet cell autoantigens are studied by investigation of the specificities of circulating antibodies present in sera derived from diabetic and pre-diabetic individuals. Significant progress has been made in the identification of T1D associated antigens, such as ICA [75], ICA69 [76], insulin [77, 78], glutamic acid decarboxylase-65 (GAD65) [79, 80], tyrosine phosphatase IA-2 and IA-2β [81, 82, 83-85], heat shock proteins [86-88], and GLIMA [74, 89].

Immune cells such as macrophages and T cells have been identified as the mediators of the pancreatic islet β-cell destruction and have been shown to act by releasing cytotoxic molecules including cytokines, oxygen free radicals, and nitric oxide (NO) [90, 91, 92]. The mechanism behind the destruction of islet β-cells mediated by T-cells has been shown in vivo in animal models; the perforin and granzymes released from the granules of cytotoxic T-cells [93] are toxic to the β-cell. T-cell induced β-cell death can also be mediated via the cell death receptors, such as Fas (CD95/APO-1) and TNFR1 [94], or by secretion of the proinflammatory cytokines, e.g. interleukin-1 (IL-1), interferon gamma (IFN-γ), and tumour necrosis factor (TNF) [95, 96]. Therefore, understanding the mechanism behind β-cell destruction mediated by T cells has helped to find ways to protect the β-cell,
aiming to prevent T1D. The vitamin B3 known as nicotinamide (NA) offers protection against toxic stimuli such as free radicals and NO by reducing their deleterious effects [97]. However, the mechanisms of T-cell-mediated islet β-cell destruction are not fully understood [98], and the triggering agent of the autoimmune response is still unknown.

Environmental factors as inducers of autoimmunity have been suggested to be involved in the pathogenesis of T1D in several studies. First, the concordance rate for T1D in monozygotic twins is less than 50% [99, 100, 101]. Second, epidemiological data and seasonality of the disease strongly indicate the contribution of environmental factors in T1D. The environmental factors can be classified into three major groups: viral infections (e.g., EV, cytomegalovirus and Rubella virus) [38, 40-44, 46, 47, 102-108], early infant diet (e.g., breast feeding versus early introduction of cow’s milk components), and toxins (e.g., N-nitroso derivatives [109-112]. In addition to these three groups, antenatal and perinatal factors [113, 114], stressful life events [115], and a combination of several environmental risk factors are associated with T1D.

Enteroviruses and Type 1 Diabetes

An association between enterovirus infections, especially CBV and T1D, has been suspected for a long time [47, 116, 117], and a number of studies have been presented. For example, increased prevalence of EV IgM antibodies have been reported in newly diagnosed T1D patients [38, 103, 118-121]. A few case studies reported that T1D developed after EV infection [104, 122, 123]. In prospective studies, EV infections have been shown to be associated with T1D, indicating that such infections can operate during the early phases of the pathogenesis [42]. In other studies EV RNA has been found more frequently in the serum and blood of T1D patients at onset [40, 41, 124] and EV infections have been shown to coincide with the appearance of autoantibodies against islet cell protein [42, 45, 125]. Moreover, it has been suggested that EV infections may initiate the process leading to β-cell destruction several years before clinical diabetes appears [42, 108, 126]. EVs have also been isolated from newly diagnosed T1D patients in a few cases [47, 105, 127, 128], and some of the isolated virus strains have been shown to cause diabetes in animal models [129, 130]. More evidence for the involvement of EV infection in T1D is the presence of EV RNA in the pancreatic islets of a few T1D patients [131].
Possible Mechanisms of EV-Induced T1D

Mechanisms of β-cell Destruction

Cell Lysis (Cytolysis)
Pancreatic β-cells can be destroyed by an acute lytic EV infection as shown in vitro in infected isolated human islets [132-135]. In a recent study, a large group of EV prototype strains were characterised according to their cytolytic activity in β-cells [134]. In addition, direct lysis of the β-cells by EV infection could be the final trigger leading to onset of clinical diabetes in a patient who already has significant loss of β-cells due to autoimmune damage.

Persistent Infection
Persistent infection with CVB in mouse islets has been shown, the persistence was associated with the development of β-cell damage [136], and EV has been detected in islet cells in a few T1D cases [137 162]. A persistent infection in islet cells could induce β-cell damage by inducing the expression of IFN-α, and subsequent expression of chemokines, leading to β-cell death [138]. The production of IFN-α has been suggested as a defence mechanism to avoid the cytolytic destruction of the islet by viral infection [139]. In vitro, a persistent CBV infection in human islets induced IFN-α expression [140]. Recently it has been shown that a CVB-4 strain able to establish a persistent infection in vitro, can induce the chemokine IP-10 during infection of human islets [141].
EV as Triggers of Autoimmunity in T1D

Molecular Mimicry

Molecular mimicry is based on a sequence homology between antigens e.g., Coxsackievirus protein 2C, and a host protein, the pancreatic islet autoantigen isoform GAD65 [65]. This homologous sequence in GAD65 has been shown to be a part of a diabetes-related T-cell epitope both in NOD-mice and humans [65, 142-144] and it induced cellular and humoral cross-reactive immune responses with the 2C region [65, 145].

Bystander Damage

Bystander activation is an alternative mechanism for the awakening of an autoaggressive T-cell response. In a mouse model it has been shown that CVB infections activate autoreactive T-cells specific for β-cell proteins [146-148], indicating that EV infection can cause β-cell damage through bystander activation.
Aims of the Study

General Aim
The overall aim of this thesis was to obtain new information of the mechanism that might be involved in the EV pathogenesis in T1D, by studying the infection of insulin producing β-cells with well defined EV strains and EV strains isolated at clinical presentation of T1D.

Specific Aims
The specific aims of this thesis were:

- To study the permissiveness of a rodent β-cell line to infection with EV (I).

- To analyze the production of the cytotoxic mediator NO in RINm5F cells during infection with strains of CBV-4 (I).

- To analyze samples from a family where the mother and one son were diagnosed with T1D on the same day (II).

- To study if the isolated EV strains could infect isolated human islets, and if such an infection induced cell death and affected the insulin release in response to high glucose (III).

- To measure if the chemokine RANTES is secreted from human islets and human islet-derived cells during infection with different EV strains (IV).

- To investigate the effect of nicotinamide on RANTES secretion from human islets and human islet-derived cells infected with EV or left uninfected.
Materials and Methods

Cell Culture

GMK Cells (I-IV)
Green monkey kidney (GMK) cells were used for tissue culture infectious dose 50 (TCID50) titrations and they were cultured in 96-well plates in Eagle’s Minimum Essential Medium (EMEM) (SVA, Uppsala, Sweden) supplemented with 10% newborn bovine serum (Hyclone, Logan, UT).

RINm5F and RCC (I)
Rat insulinoma RINm5F cells were cultured in EMEM supplemented with 10% newborn bovine serum. The RINm5F cells were cultured as monolayer cells and as RINm5F cell clusters (RCC). The RCC originated from some of the RINm5F monolayer cells, which during continuous culture, formed mushroom-like cell clusters that detached from the monolayer. The RCC were harvested from cell culture flasks and transferred to non-attach 6-well plates where they were cultured for 3–7 days before virus inoculation.

3H-Thymidine incorporation into the cellular DNA was used to determine the rate of cell proliferation in uninfected RCC.

Human Islets (III-IV)
Human pancreatic islets were obtained from brain-dead donors at transplantation units in Sweden, Norway, Finland and Denmark. The pancreatic islets were isolated in Uppsala, Sweden [149]. The islets were cultured in CMRL-1066 (Gibco-BRL, Invitrogen) supplemented with 10 mM nicotinamide (NA) (Sigma Chemicals), 10 mM Hepes buffer (Gibco-BRL, Invitrogen), 0.25 μg/ml fungizone (Gibco-BRL, Invitrogen), 50 μg/ml gentamicin (Gibco-BRL, Invitrogen), 2 mM L-glutamine (Gibco-BRL, Invitrogen), 10 μg/ml ciprofloxacin (Bayer) and 10% heat-inactivated human serum and kept at 37°C. For the virus infections the islets were cultured free-floating in
RPMI, 5.5 mM glucose, (SVA, Uppsala, Sweden) supplemented with 10% heat-inactivated human serum or foetal calf serum.

The purity of the pancreatic human islets was determined by microscopic characterization after staining with diphenylthiocarbazone. Evaluation of the quality of the islets was assessed in a dynamic perifusion system examining the response to high glucose stimulation.

Animal Models (II)
Ten female CBA/J mice and 15 DBA1 mice of mixed gender, 10–15 weeks old, were used to study infections with pooled T1 and T2. The mice were bought and maintained at the animal facility at MTC, the Karolinska Institute, Solna, Sweden

Viruses

Virus Strains (I-IV)
The virus strains used in this study all belong to the genus EV. When the RINm5F cells were infected (I), CAV-1, CBV-7 (in-house strains), CBV-3 Nancy strain, Echovirus 7 Wallace strain, and two strains of CBV-4, the E2-Yoon strain, and the prototype strain of CBV-4 (JVB) (ATCC) were used.

The virus strains used to infect the isolated human islets (T1, T2, Erik and Adrian) were isolated from T1D patients at onset and from a brother of one of the patients (III, IV). Two additional strains of EV (VD2921, V89 4557) both serotyped as CBV-4 were also included (IV). Both strains have previously been shown to infect human islets and to cause no CPE or extensive CPE, respectively, during replication in islets.

Pooled T1 and T2 were also used to infect CBA/J and DBA1 mice (II). These viruses were isolated from stool samples from a mother (T1) and her son (T2) diagnosed with T1D on the same day. The isolated strains (Adrian, Erik, T1 and T2) were used to inoculate GMK, RD, CHO, RINm5F, RCC, and HeLa cells, for tropism analyses and to study their effect on cell morphology.
Virus Infection (I-IV)
Isolated human islets, islet-derived cells (IDC), pancreatic exocrine cells, RCC and RINm5F monolayer cells were inoculated with 100 µl of virus stock diluted to 10^3 TCID50 per 0.2 ml. After infection, the cells were incubated at 37°C for 30 minutes to allow the virus to attach.

Measurement of Virus Replication (I-IV)
Viral replication was determined by TCID₅₀ titration on GMK cells cultured in 96-well plates in EMEM supplemented with 2% newborn bovine serum. Samples of culture medium were withdrawn from the virus-infected islets or other EV infected cell cultures at different time points post infection. The samples were then serially diluted and each dilution was added in triplicate to the GMK cells. The virus titre was determined by the highest dilution able to induce typical EV morphological changes to 50% of the inoculated cell cultures.

Immunostaining (I-III)
Immunostaining was performed with an EV specific antibody on HeLa cells cultured on slides and on paraffin sections of human pancreatic islets after infection with the four isolated strains: Adrian, Erik, T1 and T2 (III). The HeLa cell culture slides were fixed in acetone at 4°C, while the 5 µm human islet paraffin sections were deparaffinised in xylene, rehydrated in graded ethanol, and then washed with PBS for 10 min. Monoclonal antibodies (MAbs), raised in mice, directed against a broad-reacting epitope on the HEV capsid protein VP1 (Dako A/S, Glostrup, Denmark) were added to the slides. After washing of the slides, the binding of primary antibodies was visualised using a PicTure-Plus Kit (Zymed Laboratories Inc. San Francisco, CA, US) containing a polymer conjugate of horseradish peroxidase and Fab fragments.

Immunostaining was also used to detect the CBV receptor CAR protein on RCC and RINm5F monolayer cells (I). RINm5F cells cultured as a monolayer on culture slides and RCC added to slides pre-treated with poly-L-lysine, were fixed in acetone at 4°C, before addition of the CAR RmcB monoclonal antibody. The binding of the primary antibody was visualised with a PicTure-Plus Kit.
Tissue samples were removed from the CBA/J mice at different times post infection and prepared for immunohistochemical staining. Monoclonal antibodies recognizing T-lymphocytes (CD4 or CD8 positive), macrophages (F4/80 positive), granulocytes (Gr1) and B-cells (CD19) as primary antibodies. The secondary antibody was a rabbit biotinylated anti-rat IgG (mouse adsorbed).

**RT-PCR (I, IV)**

Reverse transcription PCR was performed to detect EV RNA (IV) and mRNA expression of CAR and β-actin (I). Total RNA was extracted from the four isolated virus strains (IV), from uninfected RCC and from uninfected RINm5F cells (I) by the use of an RNeasy mini kit (Qiagen). The RNA was stored at -70 °C until reverse transcribed into cDNA using Sensiscript Reverse Transcriptase (Qiagen) and oligo-dT primers. PCR was performed using two microlitres of cDNA and HotStarTaq Master Mix Kit (Qiagen). Specific primers for rat CAR and for rat β-actin (I) and for EV (IV) were used and can be seen in Table 1. The PCR product was visualised on a 1.5% agarose gel containing ethidium bromide. The T1 strain was subsequently genotyped by Dr. Merja Roivainen, National Public Health Institute, Helsinki, Finland.
Table 1. Primers used for EV amplification and for rat CAR and β-actin amplification

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Annealing Temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1st PCR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECBV1 (sense) [40]</td>
<td>5'-GGTACCTTTGTGCCTGTT-3'</td>
<td>53°C</td>
</tr>
<tr>
<td>ECBV5 (antisense) [40]</td>
<td>5'- GATGGCCAATCCAATAGCT-3'</td>
<td></td>
</tr>
<tr>
<td><strong>2nd PCR</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECBV1 (sense) [40]</td>
<td>5'-GGTACCTTTGTGCCTGTT-3'</td>
<td>53°C</td>
</tr>
<tr>
<td>Verheyden et al (antisense) [150]</td>
<td>5'-TTGTCACCATAAGCAGCCA-3'</td>
<td></td>
</tr>
<tr>
<td>ratCAR (sense) [151]</td>
<td>5'-CTCTCTGGGGTTGCAAATA-3'</td>
<td>54°C</td>
</tr>
<tr>
<td>ratCAR (antisense) [151]</td>
<td>5'-GGCATTCTTCTGGGAATCTG-3'</td>
<td></td>
</tr>
<tr>
<td>rat β-Actin (sense) [151]</td>
<td>5'-CACGGCATTGTAAACACTG-3'</td>
<td>54°C</td>
</tr>
<tr>
<td>rat β-Actin (antisense) [151]</td>
<td>5'-GGACCCCAGGGATGATG-3'</td>
<td></td>
</tr>
</tbody>
</table>

Studies of Cell Morphology and Viability

The morphological changes induced by the virus infection of the human islets (III, IV), IDC (IV), RCC and RINm5F monolayer cells (I) were examined every day under a light microscope. The degree of cytopathic effect (CPE) on infected islets and cells was compared to the uninfected controls and ranked from 0 to 4+, with 0 indicating no CPE and 4+ a 90–100% disruption of the cell monolayer or the RCC/islet morphology.

Human islets were also examined by electron microscopy (IV) on day three and day six post infection. Infected and uninfected islets were fixed in 2% glutaraldehyde and 1% formaldehyde, followed by 1% osmium tetroxide, then dehydrated in graded ethanol and embedded in TAAB-812-resin. Ultrathin sections (500 Å) were counterstained with uranyl acetate and lead citrate.
The viability of uninfected RCC and RCC infected with the E2 strain (I), uninfected isolated human islets and human islets infected with the four isolates (III) was performed by initial trypsinisation followed by staining with trypan blue to assess cell viability on day three and seven post infection.

Glucose Stimulation, Insulin Measurements and Blood Glucose Levels

Glucose stimulation tests were performed on human islets (III) infected with the four isolates as well as on uninfected control islets (Table 2). A number of islets were handpicked for the glucose stimulation experiments on day four and day six post infection from all culture conditions. After glucose stimulation they were kept overnight at 4°C in 200 µl acid ethanol and then frozen for subsequent analyses of proinsulin. Proinsulin was measured with a proinsulin ELISA (Mercodia AB, Uppsala, Sweden) and the proinsulin content was calculated from the standard curve in pmol/l/islet.

Table 2. Glucose stimulation of infected and uninfected human islets.

<table>
<thead>
<tr>
<th>Culture conditions (mM glucose)</th>
<th>Well</th>
<th>Day 0-3</th>
<th>Day 3-4*</th>
<th>Day 4-7</th>
</tr>
</thead>
<tbody>
<tr>
<td>A, E, T1 and T2 -1</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>A, E, T1 and T2 -2</td>
<td>5.5</td>
<td>16.5</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>Control -1</td>
<td>5.5</td>
<td>5.5</td>
<td>5.5</td>
<td></td>
</tr>
<tr>
<td>Control -2</td>
<td>5.5</td>
<td>16.5</td>
<td>5.5</td>
<td></td>
</tr>
</tbody>
</table>

* Culture medium was changed in all wells day 3 to RPMI containing either 5.5 mM or 16.5 mM glucose. It was changed again day 4 in all wells to RPMI containing 5.5 mM glucose.

Samples of the culture medium from all wells were withdrawn every day, also before and after the change of culture medium. These were used for
proinsulin and insulin measurements using a proinsulin ELISA and a high range rat insulin ELISA (Mercodia AB, Uppsala, Sweden). Insulin concentrations were calculated from the standard curve in µg/l. The insulin release in response to high glucose was then calculated by subtracting the insulin content of the culture medium on day 4 post infection from the content on day 3 post infection.

RCC and monolayer RINm5F cells (I) were trypsinised and the number of cells counted before insulin was extracted overnight at 4°C in 200 µl acid ethanol (1.5 ml of 12 M HCl added to 98.5 ml of 70% ethanol). The extracts were diluted 1:16 and insulin was measured using an Ultrasensitive rat insulin ELISA (Mercodia, Uppsala, Sweden) with a lowest detection limit of 0.02 µg/l.

Glucose tolerance tests were performed in mice (II) on different days post infection using four uninfected control mice and four mice that had been injected intraperitoneally (i.p.) with pooled T1-T2. After overnight starvation, blood glucose concentrations were monitored from the tail vein at different times. The glucose concentrations were measured directly by using sticks and a precision QID glucose sensor (Medi Sense Inc., Bedford MA 01730).

Nitric Oxide Analysis

Inoculation of RCC with Virus, IFN and Poly-IC (I)
RCC infected with CVB-4-E2 were treated with rat recombinant IFN-γ (Boule Nordic AB, Sweden) 100 U/ml, added one or 24 hours post infection. The time delay before the addition of IFNs compared to the addition of virus was to ensure the formation of viral double-stranded RNA (dsRNA) before the IFN-γ was added. As a positive control, an artificial dsRNA (poly-IC) (Sigma-Aldrich, Sweden) was added at 100 µg/ml together with 100 U/ml rat IFN-γ or at 400 µg/ml together with 500 U/ml rat IFN-γ. RCC treated with poly-IC alone, IFN-γ alone, or with no additions, were also included as controls.

Nitrite Measurements (I)
After formation, nitric oxide (NO) reacts to form the stable products: nitrite and nitrate. Therefore, the nitrite concentration was measured in culture medium samples as an indication of NO formation. Samples for nitrite measurements were collected from the culture medium of E2-infected RCC, E2-
infected and rat IFN-γ treated RCC and from poly-IC- and rat IFN-γ-treated RCC at different time points post infection. Nitrite was detected by mixing 100 µl of culture medium from the infected, treated or untreated cells with 10 µl of Griess reagent (0.5% naphthylethylenediamine dihydrochloride and 5% sulphamamide in 25% H3PO4) [152]. After a colour reaction at 60°C for 2 minutes, the absorbance was read at 546 nm. Nitrite concentrations in the samples were calculated from sodium nitrite standards diluted in EMEM.

Analyses Performed in the Case Report Study (II)

Neutralization Test (II)
To study the antibody response against CBV-4 and CBV-5 in the family, consisting of a mother and son diagnosed with T1D on the same day, and the father and a second son, serum samples were collected at onset and after two and nine months. Five strains of CBV-4 (VD2921, V89 4557, V345, JVB and E2), three strains of CBV-5 (5784, 9429 and 9699), and Ljungan virus and the T1 and T2 isolates were used. For analyses of neutralizing antibodies, 5 µl of the serum specimens were serially diluted in EMEM in two-fold steps from 1/20 to 1/2560. The diluted sera and EMEM containing 100 TCID50 of the virus per 0.1 ml were mixed and incubated for 90 min at 37°C. The serum-virus mixtures were then transferred to GMK cells cultured in 96-well plates. The titres were recorded after 4–8 days.

Antibodies against GAD65 (II)
Antibodies against glutamic acid decarboxylase 65 (GAD65) were measured with Diamyd’s Anti-GAD65 RIA (Mercodia AB, Uppsala, Sweden). Using a cut-off of 9.5 U/l the specificity was 99% and the sensitivity was 74%.

HLA Typing (II)
HLA typing of DRB1 and DQB1 genes was achieved with Dynal Classic SSP (Dynal, Norway), a PCR based method using sequence specific primers for each HLA-allele and visualisation of PCR products on a 1.2% agarose gel. The whole family was HLA-typed.

Measurements of RANTES (IV)
The secretion of the chemokine RANTES/CCL5 was studied in cultured human pancreatic islets or IDC after infection with different EV strains or in
cultures of uninfected controls. Samples were collected day one and day four post infection from cultured IDC, while samples were collected day one and day four post infection from cultured human islets. A human RANTES instant ELISA (Bender MedSystem) was used for the analysis. In addition, the RANTES content of infected and uninfected islets was measured on day seven and day ten post infection after sonication of the islets.

**Statistical Analyses**

Data are presented as mean±SEM. A p-value lower than 0.05 was considered statistically significant.

Nitrite concentration in culture medium was compared between groups with ANOVA. ANOVA and Student’s t test for independent samples were used for comparing of the viability (I). Rates of insulin release (mU/islet/24 hr) from infected and control islets, including calculated differences, were tested using Wilcoxon signed ranks test and Mann-Whitney test (III). Differences in the degree of cytolysis/islet destruction were tested with the independent samples t-test for comparing paired samples (III). The comparisons were between islets infected with one isolate and islets infected with another or left uninfected for each time point. RANTES secretion (pg/ml) from cultured islets and IDC were tested with the independent samples t-test comparing infected and uninfected samples (IV).
Results

Cell and Islet Studies

RINm5F Cells and RCC (I)

After the formation of the RCC from the RINm5F monolayer cells, experiments were performed using these as an alternative cell culture model for the β-cells to study the effect of EV infection.

Incorporation of $^3$H-thymidine into cells within the RCC was seen by autoradiography, indicating that the cells within the RCC continue to divide (Figure 1).

![Figure 1. Autoradiography of uninfected RCC labelled for 2 hours with $^3$H-Thymidine and counterstained with hematoxylin (magnification 20 x) 5 days after harvest.](image)

Immunostaining for detection of CAR on attached RCC and on RINm5F cells cultured as monolayer was performed and resulted in positive staining only of the RCC (Figure 2), although RT-PCR for rat CAR revealed that both RCC and RINm5F monolayer cells expressed CAR mRNA.
30

Figure 2. Immunostaining for CAR (magnification 20x). a: RCC positively stained for CAR; b: RCC without staining; c: monolayer RINm5F cells negatively stained for CAR.

Human Islets (III)

The isolation of human pancreatic islets resulted in a purity of 50-80% and the islet-containing fractions were determined by microscopic characterization and staining with diphenylthiocarbazone. A quality test was performed by checking the islets’ response to high glucose, before infecting them with virus. The islet batches responded to the glucose challenge with a variation between donors (Figure 3).

Figure 3. Dynamic insulin release. Isolated islets (n=20) were perifused with 1.67 (dotted line) and 16.7 (bold line) mM glucose as indicated in the figure. Data are presented as means±SEM.
Characterization of the Isolated Virus Strains

These viruses were isolated from stool samples from a mother (T1) and from her son (T2) diagnosed with T1D on the same day, and from a pair of twins (Erik) and (Adrian), at the time of diagnosis of T1D in one of the twins (Adrian). All four isolates were used to inoculate GMK, RD, CHO, RINm5F, RCC, and HeLa cells, for tropism analyses and to study their effect on cell morphology. The four strains replicated and induced morphological changes, CPE typical for EV i.e. rounding up and subsequent detachment of the cells, in GMK and HeLa cells. They also replicated in RCC and in human islets (Table 3). In contrast, none of the isolates could replicate in RD, CHO or in RINm5F cells cultured as monolayer.

Table 3. Titre increase and CPE/cell destruction of four isolated virus strains (Adrian, Erik, T1 and T2) in different cell lines.

<table>
<thead>
<tr>
<th>Cell line (titre increase /degree of CPE)</th>
<th>HeLa</th>
<th>RD</th>
<th>CHO</th>
<th>GMK</th>
<th>RINm5F</th>
<th>RCC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrian</td>
<td>5.0/0</td>
<td>-2.0/0</td>
<td>-1.5/0</td>
<td>3.5/4</td>
<td>0.0/0</td>
<td>5.5/0</td>
</tr>
<tr>
<td>Erik</td>
<td>5.0/0</td>
<td>0.0/0</td>
<td>-2.0/0</td>
<td>4.5/4</td>
<td>0.0/0</td>
<td>4.5/0</td>
</tr>
<tr>
<td>T1</td>
<td>2.5/4</td>
<td>0.0/0</td>
<td>-3.0/0</td>
<td>2.5/4</td>
<td>0.0/0</td>
<td>0.5/0</td>
</tr>
<tr>
<td>T2</td>
<td>0.5/3</td>
<td>n</td>
<td>-1.0/0</td>
<td>3.5/4</td>
<td>0.0/0</td>
<td>2.5/0</td>
</tr>
</tbody>
</table>

* Differences in log TCID₅₀ titres are presented.
* n = not done
All four isolates were positive using the Enterovision-RT-PCR-kit indicating that the four isolates belonged to the EV genus. To confirm our results we performed a semi-nested EV-RT-PCR using EV specific primers binding to the conserved 5’UTR. After the second PCR the amplification of all EV isolates revealed clear bands of the expected size 538 bp (Figure 4). In addition, the isolate T1 was sequenced and genotyped as CBV-5.

Figure 4. Electrophoresis analysis of the PCR products from human pancreatic islets infected with the isolates. 1st PCR: lane 1 show bands between 100 and 1500 bp. Lanes 2 - 5 show the PCR product from human pancreatic islets infected with T1, T2, Adrian (A), and Erik (E). 2nd PCR: lane 1 shows the DNA ladder. Lanes 2-5 show the T1, T2, A, and E amplicons.
Immunostaining for EV (III)

Immunostaining for EV on isolated human islets infected with the four isolates was positive for EV, clearly indicating production of progeny virus (Figure 5).

A.           B.

Figure 5. Immunostaining for EV in isolated human islets. A: uninfected control islets; B: islets infected with the Adrian (A) isolate three days post infection. (Magnification 60 X).

Effect of EV Infection

Viral Replication and Cell Morphology

RINm5F cells and RCC (I)

None of the CBV strains, the CAV strains, the echovirus strain, or the isolated strains replicated in the RINm5F cells cultured as monolayer. However, in the RCC formed from the RINm5F cell monolayer all EV strains replicated. The effect of IFN-γ on EV replication in RCC was a reduction of viral replication when IFN-γ was added 1 h after infection. However, if the IFN-γ was added 24 h post infection the replication was not affected (Figure 6).
Figure 6. Mean TCID50 titres of CBV-4 E2 strain in RCC, with and without addition of rat IFN-γ. hpi= hours post infection.

The EV-infected RCC were studied under a light microscope and compared to uninfected controls for signs of CPE. The infected RCC showed no signs of destruction compared to uninfected controls. In the case where viral replication was blocked by addition of IFN-γ, no difference in appearance was seen compared to uninfected RCC. When the RCC were cultured for longer periods (8–21 days) there was a more pronounced degeneration in both the infected and uninfected RCC.

Even though the RCC did not reveal severe destruction during seven days of culture, infected as well as uninfected RCC developed a denser appearance in the centre that increased with time of culture. Paraffin sections of such dense RCC cultured for 14 days showed necrosis in the centre of the cell clusters and single pyknotic cells.

Trypan blue staining for dead cells, after trypsinisation of the E2-infected RCC, showed that cell viability in the infected RCC did not differ significantly from that of the control cells three or seven days post infection.

Human Islets (III)

The isolated virus strain (T1, T2, Erik and Adrian) were used to infect human pancreatic islets. Virus TCID$_{50}$ titre increased from day three to day seven post infection indicating viral replication. Even though all four isolated EV strains were able to induce CPE/islet destruction, the degree varied with the EV strains (Table 4). All infected islets were significantly more degraded than the uninfected controls day seven post infection (p< 0.001). When paired samples statistics were performed, the most destructive isolate day seven post infection was found to be the virus obtained from the T1D
twin, Adrian, p<0.046. Some of the variations with regard to both degree of CPE and degree of viral replication might also be dependent on the islet donor.

The number of dead cells in the islets (including those detached from the islets) showed that islet cultures infected with any of the four isolates did not differ from the control islets day three post infection. On day seven post infection, however, infected islets contained more dead cells than the control islets (p<0.05).

Table 4. Degree of CPE/islet destruction in isolated human islets infected with Adrian (A), Erik (E), Tuvemo1 (T1) and Tuvemo 2 (T2) compared with uninfected control islets.

<table>
<thead>
<tr>
<th>Virus</th>
<th>n</th>
<th>Day 3</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>A -1*</td>
<td>10</td>
<td>1.7</td>
<td>2.6</td>
</tr>
<tr>
<td>A -2#</td>
<td>10</td>
<td>1.7</td>
<td>2.7</td>
</tr>
<tr>
<td>E -1</td>
<td>10</td>
<td>1.1</td>
<td>1.4</td>
</tr>
<tr>
<td>E -2</td>
<td>10</td>
<td>1.1</td>
<td>1.7</td>
</tr>
<tr>
<td>T1-1</td>
<td>10</td>
<td>1.6</td>
<td>2.3</td>
</tr>
<tr>
<td>T1-2</td>
<td>10</td>
<td>1.5</td>
<td>2.3</td>
</tr>
<tr>
<td>T2-1</td>
<td>10</td>
<td>0.9</td>
<td>1.3</td>
</tr>
<tr>
<td>T2-2</td>
<td>10</td>
<td>0.8</td>
<td>1.2</td>
</tr>
<tr>
<td>Control -1</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control -2</td>
<td>10</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* = cultured in 5.5 mM glucose during the whole culture period
# = cultured in 16.5 mM glucose day three to four post infection
Electron microscopy of human pancreatic islets infected with the isolated virus strains (Adrian, Erik or T1) six days post infection showed a recognised hallmark for EV infection, a reorganisation of ER and other intracellular membranes to generate clusters of vesicles that serve as the sites for RNA replication (replication complexes). In addition, the nuclei of islet cells infected with these isolates showed chromatin condensation, possibly indicating necrotic and/or apoptotic cells. The replication complexes induced by the infection with the Adrian strain can be seen in Figure 7A, and in higher magnification, viral particles of about 30 nm are visible inside some of the vesicles (Figure 7B). In contrast, none of this could be seen in uninfected islet cells (Figure 7C).

Together with the results showing EV specific staining of the infected human islets and increased viral titres, this shows that these strains have tropism for the β-cells in vitro.
Ultrastructural changes and progeny virus production in infected human β-cells. In islets infected with the Adrian isolate, the cells revealed indications of apoptosis as well as necrosis such as perinuclear chromatin condensation. In β-cells from such islets, virus-induced reorganisation of intracellular membranes and vesicle formation could be seen (A) magnification 20,000 X. Progeny virus (size ≈ 30 nm) can be seen in the virus replication complexes (B) magnification 80,000 X. Uninfected human islets cultured for six days (C).

IDC and Exocrine Cells (IV)

Inoculation of the IDC with the EV strains VD2921, V89 4557, and Adrian revealed that all the strains could replicate in these cells (Table 5). The infection with V89 4557 strain caused the most lytic infection, while infection with the VD2921 strain caused almost no CPE (CPE was seen only in islets from one donor) and the titre increase was also significantly lower than that of V89 4557 (p ≤ 0.005). The infection with the Adrian strain revealed a titre increase and a degree of CPE that was in between that of VD2921 and V89 4557.
Table 5. Virus titre increase and degree of CPE in cultures of human IDC infected with three different strains of EV (V89-4557, VD2921 or Adrian) seven days post infection, or left uninfected.

<table>
<thead>
<tr>
<th>Virus</th>
<th>n</th>
<th>Titre increase (^a)</th>
<th>Degree of CPE/cell destruction</th>
</tr>
</thead>
<tbody>
<tr>
<td>V89-4557</td>
<td>4</td>
<td>2.75 ± 0.63</td>
<td>3.25</td>
</tr>
<tr>
<td>VD2921</td>
<td>6</td>
<td>1.00 ± 0.85</td>
<td>0.25</td>
</tr>
<tr>
<td>Adrian</td>
<td>5</td>
<td>1.50± 0.58</td>
<td>1.25</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>0 ± 0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Virus titres were determined in culture medium from infected cell cultures

When the exocrine cells were infected with the EV strains VD2921, V89 4557 and Adrian, none of the virus strains used were able to induce any degree of CPE/cell destruction on the exocrine cells at two days post infection. At seven days post infection only the V89 4557 strain induced CPE.

In vivo (CBA/J and DBA1 Mice) (II)

Neither the CBA/J mice nor the DBA mice revealed any symptoms of infection with the pooled T1-T2 as late as 63 days post infection. From three out of ten CBA/J mice, virus could be isolated on GMK and HeLa cells. Only the suspensions from the spleen and the pancreas were positive, suggesting that these isolates had virus tropism for the pancreas in vivo.

Insulin, Proinsulin and Blood Glucose Measurements

RINm5F and RCC (I)

There was no difference in insulin content between the RCC and the RINm5F cells.
Human islets (III)
Uninfected human islets responded significantly better to glucose stimulation than islets infected with the isolated strains T1, T2, Adrian and Erik, three days post infection. There was a statistically significant difference ($p<0.04$) between islets infected with the Adrian strain and control islets and also between islets infected with the T2 strain and control islets ($p<0.05$) day three post infection with respect to the secretion of insulin in response to high glucose (Table 6).

Table 6. Insulin release in the culture medium in response to high glucose during 24 hours.

<table>
<thead>
<tr>
<th>Samples</th>
<th>n</th>
<th>Mean Value mU/l</th>
<th>Median mU/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adrian</td>
<td>7</td>
<td>1.8</td>
<td>2.2</td>
</tr>
<tr>
<td>Eric</td>
<td>7</td>
<td>1.9</td>
<td>2.4</td>
</tr>
<tr>
<td>T1</td>
<td>7</td>
<td>2.8</td>
<td>1.2</td>
</tr>
<tr>
<td>T2</td>
<td>6</td>
<td>1.3</td>
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<tr>
<td>Control</td>
<td>7</td>
<td>4.4</td>
<td>3.6</td>
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The proinsulin content in the culture media day three and day six as well as in islets day six did not reveal any differences between infected and uninfected wells.

Blood Glucose in Mice (II)
Glucose tolerance test performed 64 days post infection did not reveal any differences between mice injected i.p. with pooled T1-T2, and the uninfected control mice.
NO Production (I)

NO production was quantified by measuring the increases in nitrite concentration in the culture medium. In previous studies, synthetic dsRNA has been used to study the mechanism behind virus-induced β-cell death. If added together with IFN-γ, dsRNA induces iNOS expression and NO production in isolated islets from human and rats [153] [154] [155]. We used the RCC formed from the rat insulinoma cell line RINm5F as a model of the β-cell. Our aim was to see if the use of a real virus would induce NO production leading to cell death.

The RCC infection with the CBV-4 strain E2 did not induce more NO production than uninfected controls after seven days of culture, also IFN-γ alone did not induce any production of NO (Figure 8). Treatment of RCC with synthetic dsRNA (poly-IC) and IFN-γ, however, induced NO in a dose dependent manner (Figure 9). These results indicated that synthetic dsRNA does not mimic the dsRNA intermediate formed during viral replication.

![Figure 8. Nitrite concentration, means±SD, in medium from uninfected RCC (n=4), RCC infected with CVB-4-E2 without addition of IFN-γ (n=4) or with IFN-γ added 1 h (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.](image-url)
RANTES Production (IV)

The amount of RANTES secreted from islets infected with the EV strains Adrian or VD2921 was significantly decreased compared with uninfected control islets day four post infection (Figure 10 A), indicating that the infection reduced the secretion of RANTES. The amount of RANTES secreted on day one and four post infection was higher in islets cultured with NA (p<0.05) than in islets cultured without NA, irrespectively of whether they were infected or left uninfected (Figure 10 A, B). The fact that the viral titres were also reduced in islets cultured with NA indicates that the induction of RANTES by the NA treatment induces an antiviral state in these cells.

When the RANTES content of the islets was measured, the amount of RANTES was significantly increased in islets infected with the Adrian strain (p<0.05) compared with uninfected control islets (Figure 11), while the RANTES content in islets infected with VD2921 did not show any difference compared to uninfected control islets. This was the case when the islets were cultured without NA. In contrast, when islets were cultured with NA, the amount of RANTES increased only in uninfected islets (p<0.05).

RANTES secretion was also measured in the IDC infected with the EV strains Adrian, VD2921 or V89 4557 during 24 hours, on day one and four post infection. The results revealed no difference between infected and uninfected cells in that respect.
A. RANTES secretion day four post infection from isolated human islets cultured with and without addition of nicotinamide (NA) before infection with VD2921 and Adrian (mean values) or left uninfected.

B. RANTES secretion day one post infection from isolated human islets cultured with and without addition of NA after infection with VD2921 and Adrian (mean values) or left uninfected.
Case Report Study (II)

Neutralising (NT) Antibodies
When measuring the NT antibody titre of the whole family against several well defined EV strains and against some isolates, the son and father both had a significant increase in NT antibody titre against two strains of CBV-4, indicating a recent or ongoing EV infection. The mother revealed a significant titre rise against one of the CBV-5 strains and against her own isolate (T1). In addition, the brother showed such a titre rise against another CBV-4 strain and against CBV-5. These results show that the whole family had a proven EV infection at the time of T1D diagnosis of the mother and son.

Autoantibody
In all serum samples from the mother there were high levels of antibodies against GAD65, >198 U/l. None of the other family members had such antibodies in any of the serum samples.
HLA Typing

When the family was genotyped for HLA-DRB1 and -DQB1, the allele HLA-DRB1*04 was detected in the brother and the allele HLA-DRB1*03 was detected in the father. In addition to both these HLA alleles, the HLA-DQB1 *03/*02 allele was detected in the mother and in the T1D son. These results showed that all the family members had either one or two alleles that are linked to T1D.
Discussion

We believe in the hypothesis that EV infections are involved in the destruction of the β-cells in T1D. Thus, the aim of this thesis was to understand more about the mechanisms by which EV infection could induce or accelerate T1D. Working with both human pancreatic islets and EV strains isolated from patients at clinical presentation of T1D is a great opportunity to obtain new information leading to a better understanding of the mechanism behind EV-induced β-cell damage. We also studied EV infections in vivo by using a mouse model in one of the studies. In addition, we had access to a rat insulinoma cell line (RINm5F cells), the parental cell line, as well as the cells that had changed their growth pattern from monolayer to free-floating cell clusters. Another cell system was also studied with regard to permissiveness to EV infection, the IDC, which were formed when the human islets were allowed to attach to the surface of the wells and a monolayer of cells was formed from the islets, the islet-derived cells.

Isolation of EV strains

We isolated three strains of EV from T1D patients and one from a T1D relative at the time of clinical presentation of the disease. It is not very common that one succeeds to isolate virus at the time of clinical presentation of T1D especially not from two members of a family. Previous studies have shown that T1D can aggregate in families suggesting that genetic predisposition is a risk factor for T1D. However, only 10% of children diagnosed with T1D have an affected family member and among first-degree relatives of individuals with T1D, the risk of developing the disease is 5–6%. Thus, the genetic susceptibility can only partly explain why an individual develops T1D. Clusters of cases would strongly indicate that an infection caused or triggered the disease. Simultaneous onset of T1D in two members of a family has been described in a few other studies. The onset of T1D in a pair of twins within 14 days was reported and both of them had encountered an EV infection (Echovirus 6) [156]. In a study in south Taiwan where they investigated 22 CBV-5 isolates, four of these isolates had been obtained from patients with T1D of recent onset [157]. Our findings together with the previously reported findings show that EV can be isolated at onset of T1D and that there might be a causal relationship between some EV infections and T1D.
Virus Replication and Degree of CPE/Cell Destruction

EV inoculations of RINm5F cells cultured as monolayer and as RCC revealed clear differences in the ability of EV strains to replicate. In the cells grown as monolayer none of the strains could replicate, while in the RCC all strains replicated, indicating that EV infection can be dependent on the growth pattern of the cells. One possible explanation for this might be that the RINm5F cells lacked expression of the CAR, or that there was a different availability of the CAR protein for viral attachment in the two systems.

When the RCC were infected with CBV-4, there was no destruction of the cell clusters compared to uninfected control RCC. This could not be due to lack of viral replication in the RCC since there were high increases in viral titres in these cells. Altogether, the data suggest a kind of carrier-state persistent infection of the RCC where only fractions of the cells are infected. Infections with picornaviruses are usually very lytic events, but persistence in vitro has been shown [158-160] also in RIN cells [161].

When isolated human islets were infected with strains of EV isolated from patients at clinical presentation of T1D, there was an increase in viral titre although not until day three to six days post infection, which is later than has been shown in previous islet studies [135, 160]. This late detection of any titre increase might be due to the change of culture medium day three and four post infection. In addition, the titre rises obtained in previous studies and in study IV when human islets were infected with strains of CBV-4 were much higher than those obtained with the isolates. A possible explanation for this might be that the CBV-4 strains had been passaged several times in cell cultures, whereas the isolates were passaged only twice. The difference in replication could also reflect differences between the donors used in the different studies.

The virus replication studies show that the isolated EV strains caused a more slowly progressing infection in isolated human pancreatic islets which also fits with the viability results; day three post infection there was no difference between infected and uninfected islets. However, seven days post infection the number of dead cells were higher in the infected islets and these results are also in line with our finding that a higher degree of islet destruction was found at that-time point post infection. When HeLa and GMK cells were infected with any of the four strains, infections caused rapid cell death suggesting that these isolates displayed a slower replication cycle only in the islet cell system. Our finding that these EVs, having a well established cytolytic potential, caused a prolonged infection in human islet cells is supported by recent findings in a mouse model in vivo. EV replicated in the mouse islets without causing cell death [162]. The degree of CPE/islet destruction caused by the infection varied between the isolates and with the islet donors.
EVs are classically associated with lytic infections, but they can also establish non-cytolytic or chronic infections [136, 163]. As described before in a study where an echovirus serotype 9 was isolated from a child at onset of T1D, the degree of CPE differed from that caused by the prototype strain of echovirus 9 (Barty and Hill), when human β-cells were inoculated [132]. In our study, the Adrian isolate, from the diabetic twin, was more destructive to the isolated human islets than the strain isolated from the non-diabetic twin Erik. In addition, ultrastructural examination of islets infected with three of the isolates, revealed the presence of virus-induced vesicles containing virus-like particles in the cytoplasm of the β-cell. This clearly shows that the isolates could replicate in the insulin-producing cells. The induction of such vesicles has earlier been shown to affect the secretory pathway of infected cells [164]. In a β-cell this might affect insulin secretion.

When pooled T1 and T2 were used to infect two different mouse strains, neither of them became hyperglycaemic. However, the virus was detected in the pancreata, indicating that these viruses are pancreotropic.

When the human IDC were infected with strains of EV (V89 4557, VD2921 or Adrian) replication occurred even though the appearance of the cell monolayer did not differ much from that of the controls except when they were infected with the V89 4557 strain. This strain has also been shown earlier to cause severe destruction of isolated human islets [135]. In contrast, when primary exocrine cells were used, neither the VD2921 nor the Adrian strain replicated and just a slight increase in virus titre was obtained when these cells were infected with the V89 4557 strain. One of the purposes of studying the permissiveness of IDC to EV infection was to see if the cells surrounding the islet cells could be infected. Our finding that these virus strains replicated and caused cell destruction (V89 4557) in the IDC indicates that cells found in close contact with islet cells are indeed permissive to these infections. Our results also indicate that since primary exocrine cells were not permissive to EV, the islet-derived cell cultures do not consist of exocrine cells. These results can help us explain the route of infection.

**The Effect of EV on Insulin Release**

When the isolated human islets were infected with any of the four isolated EV strains, they showed an impaired ability to secrete insulin in response to high glucose compared with the control islets. The ability of the isolate Adrian (diabetic twin) and the isolate T2 (diabetic son) to significantly affect the β-cells’ secretion of insulin in response to high glucose as early as three days post infection, before there was any sign of dead cells, indicates a diabetogenic property in these two isolates. This shows that these viruses infect and affect the β-cell, which has partly also been shown previously [133, 135].
The Effect of EV Infection on NO Production

The addition of poly(IC) together with IFN-γ to RCC, increased the nitrite concentration in the culture medium. The intermediate dsRNA formed during viral replication together with IFN-γ, however, did not affect the nitrite concentration compared to uninfected controls. This would indicate that poly(IC) can not mimic viral dsRNA in that respect. Possible explanations for the lack of NO production in the virus-infected cells is that the infection might have caused host cell shutoff of protein synthesis, which then would have inhibited the formation of inducible nitric oxide synthesis, the enzyme responsible for NO production. The lack of NO production, in response to CBV-4 infection in the RCC does not exclude the role of a virus infection as the cause of β-cell damage in T1D, since in studies of β-cell damage in response to dsRNA and IFN-γ in rat islets and β-cells, both NO dependent [165] and NO independent [166] mechanisms have been shown.

The Effect of EV Infection and NA on RANTES Secretion from Islets and IDC

EV infection of IDC did not cause changes in the secretion of RANTES when compared with uninfected controls, indicating that these cells do not secrete RANTES under these conditions. In contrast, EV infection of islets caused a decrease in the secretion of RANTES at four days post infection when compared with uninfected controls. This can be explained by earlier published results regarding these viruses’ ability to both block the cell’s own protein synthesis and also to block the intracellular protein transport [20, 21, 167]. Also the uninfected islets seem to secrete RANTES. This is in line with earlier reports describing that the isolation of islets is a very stressful event for the islets and that such islets secrete both tissue factor and MCP-1 [168].

Most interestingly, when the islets were cultured with NA their secretion of RANTES increased irrespectively of whether the islets were infected or not. This increased RANTES secretion in EV-infected β-cells was accompanied by a decreased viral titre indicating that the NA treatment induced an antiviral state in the β-cell. An earlier study has shown effects of NA on the secretion of the chemokine IP-10 from EV-infected islets [169]. When we compared the RANTES and IP-10 secretion results from the same islets and infected with the same EV strains, we found that the NA had reduced.blocked the IP-10 secretion but, most interestingly, it had increased the secretion of RANTES. Knowing from other work, that the transcription factor NF-κB is involved in the regulation of IP-10 expression in astrocytes
and that NA is thought to interfere with gene transcription by inhibiting PARP activation of NF-κB [172], it might be that the block of EV induced secretion of IP-10 in islets after culture with addition of NA is an effect of the inhibition of PARP-mediated activation of NF-κB. Meanwhile the opposite happened with the secretion of RANTES, even though RANTES induction has been shown to depend on NF-κB activation [173]. The increased secretion of RANTES indicates that different pathways are activated in RANTES versus IP-10 induction in human islet cells and that the addition of NA only blocks one of them. It has also been shown that NA has an immune regulatory effect, since it is able to reduce spontaneous as well as in vitro-induced Th-1 like responses [174]. A Th1 to Th2 cytokine shift was demonstrated in NOD mice [175] and this could also explain why the NA effect on RANTES and IP-10 differs, implying that there might be a Th1 to Th2 chemokine shift [175] also in this system.
Conclusions

- All four isolates belonged to the enterovirus genus and revealed tropism for isolated human pancreatic islet cells.

- The isolated strains were able to affect the insulin release in response to high glucose three days post infection before the infection affected the islet cell viability.

- The synthetic dsRNA does not mimic the dsRNA intermediate formed during viral infection inducing iNOS and NO production in a β-cell line.

- EV infection of isolated human pancreatic islets decreases RANTES secretion even when the islets were infected with a non-lytic strain.

- Nicotinamide increased the RANTES secretion in isolated human pancreatic islets irrespectively of whether the islets are infected or not.
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